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HEPATIC CLEARANCE OF CANDIDA ALBICANS IN NORMAL AND CORYNEBACTERIUM PARVUM TREATED RATS

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Everett D. Benson

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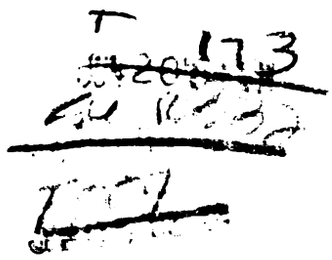

HEPATIC CLEARANCE OF *CANDIDA ALBICANS* IN NORMAL AND *CORYNEBACTERIUM PARVUM* TREATED RATS

by

Richard Trevor Sawyer

A DISSERTATION

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ABSTRACT

HEPATIC CLEARANCE OF *CANDIDA ALBICANS* IN NORMAL AND *CORYNEBACTERIUM PARVUM* TREATED RATS

by

Richard Trevor Sawyer

Hepatic clearance of *Candida albicans* from the bloodstream of rats and from perfusion medium by perfused rat livers was characterized and compared in normal rats and rats treated with *Corynebacterium parvum*. Normal rats cleared over 90% of 10^6 intravenously (i.v.) injected yeasts from the bloodstream in five minutes. All yeasts were recovered among the various reticuloendothelial (RES) organs after 30 minutes. Approximately 70% were in the liver suggesting that this organ was the major site of vascular clearance. The perfused liver, in the absence of humoral factors, trapped an average of 85% of the infused yeasts in a single pass. No organisms were killed. Trapping and killing were not enhanced by addition of 10% whole rat blood or 5% rat plasma to the perfusion medium or extending perfusion times to three hours. Scanning electron microscopic (SEM) characterization of cryofractured perfused rat livers showed *C. albicans* trapped in liver sinusoids. Yeasts were usually adhered to the

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fenestrated endothelial lining of the sinusoids, resulting in "log jams" which backed yeasts up into portal veins. Yeasts were seldom associated with Kupffer cells.

In *C. parvum*-treated rats (350 µg/rat) over 90% of 10^6 i.v. injected yeasts were cleared from the peripheral blood in 30 minutes. The percent distribution of all RES organs except the liver was essentially similar to normal rats. Only 26% of the yeasts were recovered from *C. parvum*-treated livers after 30 minutes compared with 70% in normal rats. Perfused livers from *C. parvum*-treated rats trapped 80 to 90% of *C. albicans* in a single pass. Approximately 20% of the yeasts were killed in the absence of plasma and 40% in the presence of plasma. Increasing the dose of *C. parvum* or prolonging the time of exposure to the vaccine did not enhance trapping or killing. Killing was inhibited by treatment with 1 mM phenylbutazone, 1 mM iodoacetic acid, 10.5 mg EDTA, and 10 mg crystalline silica. SEM of cryofractured livers from *C. parvum*-treated rats revealed large numbers of white blood cells, morphologically similar to lymphocytes and macrophages, adhering to portal veins and sinusoids. Treatment with *C. parvum* also resulted in a relative monocytosis. Large aggregations or yeasts clusters were trapped along portal veins and in sinusoids. Phagocytosis of *C. albicans* was easily observable.

Cumulatively these data suggest that in normal rats hepatic clearance of *C. albicans* is primarily by

nonphagocytic trapping in liver sinusoids. In *C. parvum*-treated rats, trapping involves both phagocytic and nonphagocytic parameters and significant numbers of trapped yeasts were killed. Humoral factors enhance killing in *C. parvum*-treated livers while metabolic inhibitors of macrophage activity block killing. These latter data suggest that *C. parvum* enhances nonspecific anti-*Candida* resistance by the activation of macrophages. It is proposed that the majority of activated macrophanges are blood monocyte derived and not preformed Kupffer cells.

DEDICATION

to

Kenneth Trevor Sawyer

Go wondrous Creature! mount where Science guides,
Go measure, Earth, weigh Air, and state the Tides,
Instruct the Planets in what Orbs to run,
Correct old Time, and regulate the Sun.
Go soar with Plato to th' empyreal Sphere,
To the first Good, first Perfect, and first Fair;
Or tread the mazy round his Follow'rs trod,
And quitting Sense call Imitating God,
As Eastern Priests in giddy Circles run,
And turn their heads to imitate the Sun.
Go, teach Eternal Wisdon how to rule;
Then drop into Thy-self, and be a Fool!

—A. Pope

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INTRODUCTION

There are a variety of nonspecific host defense systems which are remarkably efficient in removing microorganisms from the bloodstream. These systems are nonspecific in that they do not usually require previous exposure to the microbe in order to function effectively and provide an initial host response toward circulating microbes (Rogers, 1960).

The mononuclear phagocyte system (MPS) represents one of these host defense systems. The MPS is composed of the peripheral blood macrophage, and the fixed tissue macrophage system. This system includes the lung alveolar macrophage, microgleal cells of the central nervous system, peripheral lymph node macrophage, splenic macrophage, bone marrow macrophage, and liver Kupffer cells (van Furth et al., 1972; Carr, 1973). Recent evidence challenges the blood monocyte origin of the fixed tissue macrophage system (Volkman, 1976).

Another host cell which removes circulating microbes is the polymorphonuclear leukocyte (PMN). These short lived, highly phagocytic cells are excluded from the blood

monocyte and tissue macrophage system (van Furth, 1973; van Furth et al., 1972).

Bloodstream clearance of intravenously injected microorganisms results in the accumulation of large numbers of these microbes in the spleen and liver (Rogers, 1960). A major group of fixed macrophages, the Kupffer cells, reside in liver venous sinusoids. It is generally thought that Kupffer cells account for most of the trapping and killing capacity of the liver (Aschoff, 1924; Baine et al., 1974; Bonventre and Oxman, 1965; Howard, 1961; Howard and Wardlaw, 1958; Jeunet et al., 1967, 1968, 1969; Manwaring and Coe, 1916; Manwaring and Fritschen, 1923; Rogers, 1960).

Moon et al. (1975) distinguished experimentally between the bactericidal and bacterial trapping functions of the liver by using an isolated perfused liver model. It was shown that the rate of *Salmonella typhimurium* trapping is essentially the same in the presence or absence of humoral factors and that Kupffer cells require humoral factors for killing to take place. In a subsequent study Friedman and Moon (1977) showed that the Kupffer cell-specific toxin, crystalline silica, enhances host susceptability to infection by the destruction of these macrophages.

The primary objective of this study is to characterize the initial clearance of *Candida albicans* by hepatic

tissue. Bloodstream clearance of *C. albicans* results in the accumulation of large numbers of yeast cells in the liver (Baine et al., 1974; Iannini et al., 1977; Louria et al., 1963). An experimental approach, similar to that of Moon et al. (1975) is used to determine whether hepatic clearance of *C. albicans* in normal rats occurs in a manner analogous to that of bacterial clearance. A second objective is to determine whether hepatic tissue can be nonspecifically enhanced in its ability to kill cleared yeasts. Hepatic clearance of *C. albicans* in *Corynebacterium parvum*-treated rats was compared to clearance of the yeast in normal rats. These objectives were evaluated by both *in vivo* and *in vitro* pathophysiological studies centered around experimental manipulation of the perfused rat liver model. Observations made in these experiments were extended and clarified by a scanning electron microscopic characterization of both normal and *C. parvum*-treated rat liver, with or without *C. albicans*.

LITERATURE REVIEW

Candida albicans is considered to be the primary etiologic agent of a collection of clinical entities known as candidiasis. This yeast is a member of the Form-class Deuteromycotina, Form-order Blastomycetes, Form-family Cryptococcaceae (Kreger-von Rij, 1973). It is a normal flora inhabitant of the alimentary tract and mucocutaneous tissues in 10% to 30% of healthy individuals (Marples and Somerville, 1968). Rippon (1974) suggests that at some point in life all persons are exposed to and temporarily colonized by this yeast.

The genus *Candida* has been reviewed by Skinner and Fletcher (1960). In terms of its' chemical composition the most remarkable aspect of this genus is the presence of a trilaminar cell wall (Bakerspigel, 1964; Gale, 1963; Al-Doory and Baker, 1971). Meister et al. (1977a) described the chemical composition of the *C. albicans* cells walls. It is composed of mannan, glucan, a small amount of ribose, chitin, and trace undefined proteins. It was found that the inner and outer layers of the cell wall are composed primarily of mannan while the middle layer

contains most of the glucan. Glucan and mannan represent the two major antigenic components of *C. albicans* yeast and blastospore cell walls (Meister et al., 1977a, 1977b).

The saprophytic and infectious state of *C. albicans* is the yeast call and its' sexual bastospore (Simonetti and Strippoli, 1973). Once infection is established in the host yeast cells germinate into the characteristic tissue state pseudohyphae, which also reproduces *in vivo* asexually.

Examining the list of factors predisposing the patient to *C. albicans* infections it becomses obvious that this yeast is an opportunistic pathogen. The opportunistic nature of infections due to the yeast has been reviewed by Chick et al. (1975), Hildick-Smith et al. (1964), and Klainer and Beisel (1969). Rippon (1974) defines four conditions predisposing patients to *C. albicans* infection including *i*. extreme youth, *ii*. drastic physiological changes such as those associated with pregnancy or diabetes mellitus, *iii*. prolonged chemotherapy, and *iv*. general debility which includes neoplasia, genetic disease and metabolic disorders. Of concern to modern hospital practice are the latter two categories.

It has been known for years that patients on prolonged anti-bacterial antibiotic therapy are prone to over colonization of the gastrointestinal tract by *C. albicans*. Overgrowth of the alimentary tract results in either

genitourinary candidiasis (Wise et al., 1976; Miles et al., 1977) or gastrointestinal candidiasis (Eras et al., 1972). The mechanism by which antibiotics predispose the patient to candidiasis have been reviewed (Seelig, 1966a, 1966b). Various chemotherapeutic drugs increase the susceptibility of the patient to *C. albicans* infection. They include drugs used in immunospuuressive therapy for neoplastic disease such as cyclophosphamide, methyltrexate and the corticosteroids (Salit and Hand, 1975). In a study of 261 autopsied leukemia patients Baker (1962) found a direct correlation between the 15% incidence of complicating fungal infections and the use of steroids, antileukemia drugs, and neutropenia. The predisposing condition of prolonged chemotherapy merges with that of general debility as a predisposing condition. Most patients with debilitating diseases receive some type of chemotherapy.

The literature shows that among patients with myeloproliferative diseases, notably acute leukemias of lymphoma, there is an increased incidence of fungal infections due primarily to *C. albicans*, *Aspergillus* spp., and *Mucor* spp. (Bodey, 1966; Eras et al., 1972; Gruhn and Sanson, 1963; Sidransky and Pearl, 1961). Hersh et al. (1965) stated that the major cause of death in 44 acute leukemia patients was infection and hemorrhage. During this ten year study fatal Staphylococcal infection decreased

from 24% to 3% and was replaced by fatal fungal infections. Three fourths of these fatal mycoses were due to *Candida* spp.

There is an abnormally high incidence of *C. albicans* infections among patients with hereditary diseases including those with cellular immunodeficiency disorders and phagocytic disorders (Baboir, 1978a, 1978b; Bellanti and Dayton, 1975; Good, 1976; Shuster and Eisen, 1976). For example, Kirkpatrick and his colleagues (1971) studied twelve patients with chronic mucocutaneous candidiasis and correlated the incidence of this disease with defects in cellular immunity, especially lymphocyte dysfunction. The incidence of *C. albicans* infections in patients with chronic granulomatous disease or myeloperoxidase deficiency can be traced to the inability of their meutrophils to kill ingested yeast cells (Baboir, 1978b; Lehrer, 1970).

A recently recognized category of predisposing factors contributing to *C. albicans* infections is that of iatrogenic procedures resulting in the introduction of normal floral yeast cells directly into the patients' bloodstream or urinary tract. Patients receiving parenteral hyperalimentation (Curry and Quie, 1971; Maki et al., 1977) and bladder catheterization (Williams et al., 1971) are most often troubled by *C. albicans* infections. Also included in this category should be drug addicts. In evaluating 77 cases of endocarditis in heroin addicts Ramsey et al.

(1970) found that next to *Staphylococcus* spp. and *Streptococcus* spp., *Candida* spp. were most commonly the etiologic agents.

Conditions which predispose the patient to opportunistic invasion by *C. albicans* have become well defined. The virulence factors possessed by this yeast are poorly understood. *Candida albicans* is not an invasive microbe. It is non-motile, lacks an antiphagocytic capsule, and when it is phagocytized it is readily killed (Lehrer and Cline, 1969). A "toxic-like death" is a uniform feature of the intravenous injection of large numbers of viable *C. albicans* into experimental animals (Hansenclever and Mitchell, 1963). Various toxic fractions isolated from cell walls of the yeast are, at best, weakly toxic and require large doses to kill experimental animals (Cutler et al., 1972; Hansenclever and Mitchell, 1962, 1963; Isenberg et al., 1963a, 1963b). Toxic substances have been found in *C. albicans* culture filtrates (Mankowski, 1962, 1968), and the yeasts' cytoplasm (Chattaway and Odds, 1971). Holder and Nathan (1973) showed that a cell free sonicate of *C. albicans* caused thrombocytopenia and clotting disorders in mice similar to those observed when mice were injected with viable yeasts. Nosal and Menyhardtova (1976) isolated a cell wall glycoprotein from *C. albicans* which causes a dose-dependent release of serotonin and decreases the

spontaneous sedimentation-aggregation rates of isolated platelets.

Iwata and his colleagues isolated and purified a glycoprotein toxin from a strain of *C. albicans* which caused fatal meningitis. It is unknown whether this glycoprotein toxin, Canditoxin, is the same as that described by Mankowski (1968), Nosal and Menyhardtova (1976) and Svec (1974). The isolation and purification of Canditoxin, its possible role in experimental *Candida* infection and its *in vitro* and *in vivo* mode of action has been reviewed (Iwata, 1977; Iwata et al., 1974, 1975). It should be pointed out that Canditoxin displays its toxic effects over a narrow host range and it is unknown whether or not all strains of *C. albicans* have Canditoxin.

In man, *C. albicans* elicits a bewildering variety of about twenty clinical entities. The diseases may be broadly categorized as either infectious diseases of cutaneous, mucocutaneous or systemic tissues, or allergic disease both of the delayed and immediate type (Rippon, 1974). In most instances infections due to *C. albicans* are treatable. Under conditions advantageous to the yeast systemic infection may occur. Systemic candidiasis involves single and multiple host organ systems. Established systemic candidiasis is refractory to therapy and has a 75% incidence of mortality.

The incidence of systemic candidiasis is increasing especially in the compromised patient. This is clearly emphasized in studies by Hammerman et al. (1974), Hutter and Collins (1962), Myerowitz et al. (1977), Louria et al. (1962) and Tola et al. (1970). In a recent study Myerowitz et al. (1977) found that in 39 proven cases of disseminated candidiasis in patients with acute leukemia, all had gastrointestinal infection. Seventy five percent of these patients had hepatic involvement and 94% had splenic involvement. This observation is contrasted by those of Louria et al. (1962) who cited the kidney as the organ most often involved during systemic candidiasis.

Even in the face of the high mortality rates associated with systemic disease newer, more aggressive, therapeutic regimes have had some success in treatment (Goldstein and Hoeprich, 1972; Tassel and Medoff, 1968). Noteworthy among the newer approaches to therapy are the successes achieved using the dialyzable lymphokine transfer factor (Buckley et al., 1968; Feigin et al., 1974; Pabst and Swanson, 1972; Rocklin et al., 1970; Valdimarsson et al., 1972).

In light of the fact that *C. albicans* infections are primarily opportunistic it is obvious that the normal uncompromised host is quite efficient at preventing infections due to this yeast. During systemic candidiasis the internal host defense systems and their response to

C. albicans are extremely important to the final outcome of the host-parasite interaction. The internal host defense systems which resist yeast invasion may be broadly divided into humoral mechanisms and cellular mechanisms. At each level of resistance there are specific and non-specific components of the defense mechanism which play a role in anti-*Candida* resistance.

The antigenic composition of *C. albicans* is quite complex, especially since both yeast and mycelial forms are exposed to the hosts' immunological tissues. Axelsen (1971, 1973) employing quantitative immunoelectrophoretic methods demonstrated 78 water soluble antigens from one strain of *C. albicans*. Yeast cells elicit strong, specific antibody responses in man and experimental animals. Hansenclever and Mitchell (1961) initially divided strains of *C. albicans* into two large serological groups, A and B, using agglutination reactions. Taschdjian and her colleagues (1964a, 1964b) described the prognostic value of precipitating antibodies present in patients with systemic candidiasis. In addition to eliciting production of precipitating, agglutinating and complement fixing antibodies (Kaufman, 1976; Merz et al., 1977), Mather et al. (1977) recently demonstrated elevated levels of homocytotropic immunoglobulin E anti-*Candida* antibody in patients with vaginal and systemic candidiasis.

Hard and Drake (1953) were unable to passively immunize rabbits using immune rabbit serum. Mourad and Friedman (1968) were able to confer protection against lethal challenge in unsensitized mice given hyperimmune mouse serum. While this might suggest a protective role for antibody in anti-*Candida* resistance, the enhanced resistance of mice receiving hyperimmune serum could also be explained by the transfer of effectors of specific cell-mediated immunity along with the "protective" antibody. Such an explanation would not have been possible in 1968. Rippon (1974) states that, other than exerting an opsonic effect, specific antibody plays a weak protective role by itself in resistance to *C. albicans*.

More important to the host are the variety of non-specific anti-*Candida* factors which occur in normal serum of man and experimental animals. Roth and Goldstein (1961) showed that a heat stable component of normal adult serum inhibited the reproduction of *C. albicans in vitro*. Hendry and Bakerspigel (1969) and Esterly et al. (1967) found that serum transferrin in its unsaturated form inhibited yeast growth. Louria and his colleagues (1972; Smith and Louria, 1972) described a heat stable, trypsin sensitive protein, "Clumping Factor" which is not antibody or complement, and which clumps *C. albicans* and *C. stellatoidea*. Morelli and Rosenberg (1971) suggested that *C. albicans* activates complement by the classical

pathway. They found that complement deficient CF-1 mice were significantly more susceptible to lethal intravenous challenge than were normal mice of the same strain. Ray and Waepper (1976) and Sohnel et al. (1976) found that *C. albicans* also activates complement through the properdin pathway.

The two components of cellular resistance to *C. albicans* are the specific cell-mediated immune (CMI) responses of the host toward the yeast, and the nonspecific phagocytic cells of the MPS and the polymorphonuclear leukocyte (PMN). The fundamental interactions of lymphocytes, macrophages and target cells which results in the expression of specific antimicrobial CMI to infection have been reviewed (Mackaness, 1971; North, 1974; Oppenheim and Seeger, 1976; Roelants, 1977; Rosenstreich and Oppenheim, 1976). Saba (1970) and Stossel (1974a, 1974b, 1974c) have reviewed the fundamental aspects of phagocytosis.

There are as yet no definitive studies, using either *in vitro* or *in vivo* models, clearly demonstrating the ability of lymphocytes and macrophages to express specific anti-*Candida* CMI. The role of CMI in host resistance to *C. albicans* infection is strongly suggested by the results of several investigations.

Salvin et al. (1965) studied the ability of neonatally thymectomectomized Swiss mice to resist sublethal

C. albicans challenge. Mice injected four weeks post-partum had increased numbers of viable cells in liver, spleen, and kidneys when compared to yeast population in organs of normal mice. All of the control animals survived intraperitoneal challenge while the thymectomized mice died by the tenth day. It was concluded that the lymphocytes, removed by thymectomy, play an essential role in resistance to *C. albicans*.

Fisk et al. (1974) found that a crude antigen from three *C. albicans* strains, a cell wall antigen, and a cytoplasmic antigen all stimulated the *in vitro* blastogenic incorporation of ^{14}C-thymidine into peripheral blood lymphocytes from ten patients with verified *C. albicans* infections. Lymphocyte transformation in response to specific antigen is considered to be a correlate of CMI (Oppenheim and Schecter, 1976).

Patients with chronic mucocutaneous candidiasis present the strongest case favoring a role for CMI in anti-*Candida* resistance. Patients with chronic mucocutaneous candidiasis have defects in their ability to mount a CMI response. Such defects can be augmented or even restored using immunotherapy with specific transfer factor (Kirkpatrick et al., 1971).

Studies on the role of thymus dependent CMI in resistance to systemic candidiasis have been preformed in congenitally athymic (nude) mice. Both Cutler (1976) and

Rogers et al. (1976) found that nude mice are more resistant to lethal intravenous challenge with *C. albicans* than their normal littermates. Rogers et al. (1976) found that when the nude mice were reconstituted by a transplant of a functional thymus, from normal syngenic mice, they became just as susceptible to intravenous challenge as normal mice. They concluded that thymus dependent CMI was associated with a depression in resistance. Cutler (1976) concluded that enhanced resistance in nude mice is due to a highly stimulated MPS.

In addition to the *in vitro* expression of correlates of CMI, such as lymphocyte transformation and production of migration inhibition factor, another feature of CMI is the ability of lymphocytes or lymphocyte supernatants (lymphokines) to transfer CMI correlates *in vitro* and *in vivo*. One such lymphokine is transfer factor (Ascher et al., 1976). Rifkind et al. (1976a, 1976b, 1976c, 1977) isolated transfer factor against *C. albicans* from sensitized mice and successfully demonstrated the transfer of specific CMI to nonsensitized mice. It was concluded that transfer factor from antigen sensitized splenic cells rendered uncommitted lymphocytes in nonsensitized mice immunocompotent with respect to *C. albicans*. This experimental animal model has been preceeded by several years of similar studies in man using transfer factor.

Recently Johnson et al. (1977) found that mice bearing the L 1210 lymphocytic leukemia tumor are significantly less resistant to systemic *C. albicans* infection. L 1210 cells suppressed the inflammatory response of PMN toward *C. albicans*. This study demonstrated the marked susceptability of the leukemic host to *C. albicans* infection. It also suggests that the neoplastic lymphocyte is not only compromised in its ability to function as a mediator of cellular resistance but it may actually suppress nonspecific PMN-mediated resistance. Taken together these studies suggest that lymphocytes do participate, probably as effectors of macrophage anti-*Candida* reactivity, in host resistance to *C. albicans*. The mechanism of this interaction is still unclear.

The nonspecific arm of cellular resistance is the subject of extensive evaluation with respect to the ability of the host to resist systemic *Candida* infection. The first type of cell evaluated for its ability to phagocytize and kill *C. albicans* is the PMN. The PMN is an efficient killer of *C. albicans*. It is currently believed that this cell represents the major component of cellular resistance to systemic candidiasis.

Lehrer and Cline (1969) studied the interaction of *C. albicans* with human peripheral blood PMN to evaluate the role of serum in phagocytic killing of the yeast. They showed that normal PMN rapidly ingest and kill

yeasts after a 60 minute exposure. Addition of serum to the test system increased the ability of PMN to kill yeasts. This enhancing effect was heat labile and did not enhance intracellular killing while phagocytosis and killing were susceptible to inhibitors of oxidative metabolism but not susceptible to inhibitors of ribonucleic acid or protein synthesis. In a subsequent study Lehrer (1970) showed that PMN from patients with myeloperoxidase deficiency disease and chronic granulomatous disease are unable to kill ingested *C. albicans*. Zeya and Spitznagel (1966) demonstrated that cationic proteins isolated from PMN lysosomes are able to kill *C. albicans*. Lehrer (1969) found that the myeloperoxidase-H_2O_2-halide ion system is candidacidal *in vitro*. Lehrer (1970) found that the myeloperoxidase deficiency disease PMN is unable to kill the intracellular yeasts although the phagocyte did prevent the yeast from formation of pseudohyphae. The chronic granulomatous disease PMN is unable to either kill intracellular yeasts or prevent pseudohyphae formation. It was concluded that the PMN lysosome possesses more than one candidacidal factor and that the major component of the intracellular killing system for *C. albicans* is the myeloperoxidase-H_2O_2-halide ion system originally described by Klebanoff (1968, 1972; Klebanoff and Hamon, 1975). Presence of a second candidacidal mechanism in human PMN, independent of the

myeloperoxidase system, was confirmed using PMN from myeloperoxidase deficient patients (Lehrer, 1972).

Lehrer (1969) found that normal serum did not inhibit the growth of *C. albicans in vitro*. Davies and Denning (1972) suggested that the ability of serum to inhibit the growth of yeast cells is due to the rapid production of germ tubes and subsequent clumping of yeasts. The ability of serum to mediate PMN killing of *C. albicans* was also studied by Denning and Davies (1972). They found that autologous plasma enhanced PMN killing of yeasts. If immune plasma, containing anti-*Candida* antibody, was absorbed with *C. albicans* mannan no decrease in killing occurred. When plasma was heat inactivated or absorbed with zymosan, which abolishes the properdin pathway, a significant reduction of PMN killing was observed. They concluded that heat labile complement components are important to, but not essential for, PMN killing of *C. albicans*. Subsequently it was found that the cell wall mannan is highly chemotactic for PMN while cytoplasmic antigens are not chemotactic (Davies and Denning, 1973). The importance of complement in PMN killing of *C. albicans* was also demonstrated in a study by Schmid and Brune (1974). Cutler (1977) found that certain strains of *C. albicans* release, into culture medium, heat stable substances chemotactic for guinea pig PMN *in vitro*. He found that the most virulent strains of yeasts are unable to release

these chemotactic factors. This might explain observations that certain avirulent *C. albicans* strains initiate strong PMN inflammatory responses while the more virulent strains do not (Albano and Schmitt, 1973).

Venkataraman et al. (1973) studied phagocytosis and killing of *C. albicans* in normal and immune serum. Optimal phagocytosis and killing occurred when immune serum was employed. While all of these studies use supravital staining and quantitative viability counts to measure PMN candidacidal activity, Yamamura et al. (1976) developed a more sensitive ^{51}chromium release assay for phagocytic killing of *C. albicans* by PMN.

Note should be taken of studies by Lehrer and Cline (1971) and Rosner et al. (1970). Both studies evaluated the candidacidal activity of PMN from patients with neoplastic disease. Lehrer and Cline (1971) found that PMN from patients with acute myeloblastic leukemia, Hodgkins' Disease, and metastatic carcinoma have impaired candidacidal activity, low levels of myeloperoxidase, and decreased PMN mobilization. Rosner et al. (1970) found that PMN from 19 acute myeloblastic leukemia patients ingest *C. albicans* at normal rates but the yeasts killed the phagocyte. This inability to kill ingested yeasts is attributed to a lysosomal defect of an unspecified nature.

Leijh et al. (1977) studied the kinetics of phagocytosis and killing of *C. albicans* by human PMN and blood

monocytes. Ninety six percent of yeast cells added to the incubation medium were ingested within the first 30 minutes of *in vitro* exposure to PMN. Pagocytosis is blocked by addition of 1 mM iodoacetic acid to the culture medium. During the next 30 minute period over half of the ingested yeasts are killed by PMN leukocyte. Intracellular killing is blocked by addition of 1 mM phenylbutazone to the culture medium. Autologous serum was employed in all *in vitro* systems, and it was shown that complement dependent opsonization of yeasts was responsible for the enhancing effects of serum on PMN function.

Solomkin et al. (1978) investigated the opsonic requirements of human PMN for *C. albicans*. The assay system in this study employed yeast cells labeled with ^{3}H-adenine, differential Ficoll-Hypaque centrifugation methods, and phagocytosis inhibition studies to show that several normal serum components participate in the opsonization of *C. albicans*. Normal human sera contains low titers (c.a. 1:40) of heat stable immunoglobulin, titers which are elevated in sera from patients with chronic *Candida* infection. It was also shown that complement and properdin activated complement opsonins amplify the opsonic activity of the heat stable immunoglobulin.

Cumulatively these studies summarize critical points concerning the nonspecific candidacidal activity of the

PMN. Function of the PMN occurs optimally at 37 C over a short period of time in the presence of complement generated opsonized yeasts. The major intracellular killing system present in the PMN, for *C. albicans*, is the myeloperoxidase-H_2O_2-halide ion system, although other systems are present (Drazin and Lehrer, 1977). Less well defined are the candidacidal functions of macrophages. *In vitro* studies on the interaction of *C. albicans* with macrophages have been restricted to investigating the function of peritoneal exudate cells (PEC), alveolar macrophages (AM), and more recently the blood monocyte (BM).

Taschdjian et al. (1971) used an immune fluorescence technique to study intracellular events taking place between PEC and phagocytized *C. albicans*. They observed that once yeasts are inside the phagolysosome the outer cell wall glucomannan proteins are stripped away resulting in the loss of immunofluorescence. The resulting yeast is the "first stage spheroplast". It is highly labile to rupture of its cell membrane and release of cytoplasmic contents. This might allow the macrophage to process these antigenic substances. This model was used to explain why copious amounts of precipitating antibody to yeast cytoplasmic antigens are produced during *C. albicans* infections in man and experimental animals.

Salvin and Cheng (1971) found that PEC, isolated from guinea pigs injected intraperitoneally with *C. albicans*, in the presence of sensitized lymphocytes and *C. albicans* soluble filtrate antigen, are inhibited in their ability to migrate on an agar surface toward heat killed yeast cells. These macrophages ingest fewer yeast cells than did PEC from normal animals. It was postulated that these less mobile, less phagocytic, yet more destructive macrophages from sensitized animals would be functionally altered in order to limit the spread of the infection through tissue. They render the host more resistant to infection. Employing time lapse phase contrast cinemicrography in an examination of the interaction of *Candida* sensitized PEC with merthiolate killed yeast cells, Neta and Salvin (1971) proposed that and initial event in specific CMI to *C. albicans* is a reduced activity of the sensitized macrophage. PEC used in both these studes were isolated from guinea pigs injected intraperitoneally with live *C. albicans*. The *in vitro* response of these PEC might not be analogous to their initial response in the peritoneal cavity towards a live yeast cell.

Ozato and Uesaka (1974) isolated mouse PEC and cultivated them *in vitro* with viable *C. albicans*. It was found that phagocytized yeast cells are unable to incorporate ^{3}H-uridine or ^{3}H-leucine for 2 h after ingestion. External yeast cells incorporate both precursors and

produce germ tubes. Within the PEC yeast cells germinate, produce germ tubes after 3 h, subsequently rupture the macrophage membrane, and elongate becoming pseudohyphae. It was concluded that the unsensitized PEC is able to phagocytize *C. albicans* but it is unable to permanently suppress the ability of the yeast to metabolize. Ultimately the yeasts kill the macrophage. It was also pointed out that the unsensitized PEC will inhibit early metabolism in the yeast. This inhibition might be enhanced in the sensitized PEC.

Employing an *in vitro* system and radiolabeled *C. albicans*, Viken and his colleagues (1974; Odegaard et al., 1974; Viken and Odegaard, 1974) studied the ability of human peripheral BM to degrade yeast cells during the *in vitro* differentiation process of becoming macrophages. It was found that functional changes in phagocytic ability occur along with *in vitro* morphological alteration. Phagocytosis and digestion of yeasts are dependent on the age of the macrophage. Optimal response occur at peak differentiation of the BM into a macrophage.

Interaction of *C. albicans* with rabbit AM has been studied by Arai et al. (1977) and Peterson and Calderone (1977). Peterson and Calderone (1977) found that AM inhibited germ tube formation, intracellular yeast macromolecular synthesis, and killed a significant portion of yeast cells over an eight hour period *in vitro*. Arai

et al. (1977) found that AM phagocytized *C. albicans* at the same rate as PMN, over a 60 minute assay period. The AM were unable to kill yeast cells. Normal serum and hyper-immune rabbit serum had no effect on the ability of AM to phagocytize and kill yeasts. After 12 hours most of the internalized yeasts had produced pseudohyphae killing the AM.

The various macrophages comprising the MPS represent a functionally heterogenous population of phagocytic cells (Walker, 1976). The studies described above indicate such functional differences in the ability of macrophages to kill intracellular *C. albicans*. Variation in the candidacidal potential of various phagocytic cells has been reviewed (Howard, 1977). The literature concerning PMN killing mechanisms has expanded over the last ten years and these mechanisms have been reviewed (Baboir, 1978a; DeChatelet et al., 1975; Saba, 1970). Data concerning the mechanisms by which macrophages kill phagocytized fungi is, at best, sketchy.

For example, Lehrer (1975) studied the fungicidal mechanisms of human BM. He found that normal human BM ingested and killed *C. albicans* and *C. parapsilosis*. Both functions are absent in BM from patients with myeloperoxidase deficiency disease and chronic granulomatous disease. Normal BM are able to iodinate heat killed intracellular *C. albicans*, a process inhibited

by phenylbutazone, methimazole, isoniazid and aminotriazole. It was found that BM, in addition to the myeloperoxidase-H_2O_2-halide ion fungicidal system, contain cationic proteins which kill *C. albicans*. It was shown that the BM metabolic response during phagocytosis is accompanied by: *i.* augmentation of oxygen consumption, *ii.* increased hexose monophosphate shunt activity, and *iii.* the ability to iodinate ingested *C. albicans*. Blood monocytes from man, mice, rats and guinea pigs but not rabbits have myeloperoxidase activity (Lehrer, 1975; Widmann et al., 1972). Kupffer cell lysosomes, in addition to myeloperoxidase, contain beta-glucuronidase, acid deoxyribonuclease (Berg and Bomun, 1973) and other hydrolytic enzyme activities (Smith and Filkins, 1971).

Recent studies indicate that phagocytosis is not an obligatory process of macrophage antimicrobial activity. The majority of work in this area has centered on macrophage cytotoxicity for cells other than microorganisms. It is apparent that macrophages release cytotoxins in the presence of target cells (Bust et al., 1974; Ghaffar and Cullen, 1976; Kramer and Granger, 1972; Lohman-Matthes et al., 1973; Melson et al., 1974). Recent work on macrophage cytotoxicity by Hemsworth and Kochan (1978) indicates that Am and Kupffer cells release antibacterial fatty acids *in vitro* and into the bloodstream of immunologically stimulated animals, independent of phagocytosis.

The ability of macrophages to release cytotoxic substances has been reviewed (Lohman-Matthes, 1976). In addition to macrophage cytotoxicity lymphocytes release cytotoxic lymphokines upon exposure to target cells (Cerottini and Brunner, 1977). Pearsall et al. (1973) found a heat labile lymphotoxin that killed *C. albicans*. Salvin et al. (1974) detected two *C. albicans* growth inhibitory lymphokines in serum from mice given BCG intravenously. These studies employ primarily *in vitro* experimental models. During *in vivo* exposure of yeasts, either in normal or immune animals, cytotoxic factors may or may not function in a manner analogous to their *in vitro* mode of action.

There are a number of *in vivo* experimental animal models evaluating the host-parasite interaction with *C. albicans*. Early studies compared the pathogenicity of the yeast phase to mycelial phase *C. albicans* (Gresham, 1966). They emphasized the importance of the acute inflammatory response to yeast cells associated with initial tissue invasion. Also emphasized in these studies is the ability of yeast cells to localize in kidney tissue. For the most part early studies on host-parasite interactions emphasized the histopathological aspects of infection, primarily in the kidneys.

Louria et al. (1960) evaluated the influence of cortisone treatment of systemic *C. albicans* infection in mice. Employing histopathological techniques and

quantitative organ distribution methods this study described the progressive pathology of renal candidiasis. While they emphasized the pathogenesis of *Candida*-kidney and *Candida*-PMN interactions in steroid terated mice this study virtually ignored the ability of the liver and lungs to eliminate the majority of the challenge dose by 14 days in both normal and steroid treated mice. Recently Wheeler and Stock (1976) and Oblack and Holder (1976) found that mice given large doses of *C. albicans* intravenously do not have metabolically compromised kidney function, even in the face of extensive tissue invasion and colonization.

Young (1958) studied tissue invastion of mice injected intraperitoneally with *C. albicans* to compare this route of injection to intravenous inoculation. This route of injection establishes chronic renal involvement. Mourand and Friedman (1961) compared routes of injection, dose responses, and comparative virulence of 37 *C. albicans* strains. A high degree of strain variation with respect to virulence was observed. Both studies emphasized that acute systemic candidiasis results from intravenous injection of viable yeast cells or cell free toxic extracts. Acute disease is dependent on a large dose of injected cells. Louria et al. (1963) compared the pathogenesis of experimental murine candidiasis using virulent and avirulent *C. albicans* strains. It was emphasized that for all six strains tested the kidney is the only tissue in

which infection progresses. The more virulent strains grew out of viable PMN after phagocytosis. Their data indicated that the majority of the challenge inoculum, located in liver, spleen and lungs, was rapidly eliminated.

Hurley (1966) studied experimental *C. albicans* infections in normal mice, mice treated with 160-495 r whole body X-irradiation, mice with alloxan induced diabetes, and mice simultaneously infected with *Proteus morganii*. As expected all of the modified hosts were more susceptible to fatal infection. She emphasized that many organ systems were involved in candidiasis of the modified host, not just kidneys.

Recently an experimental guinea pig model has been developed (Hurley and Fauci, 1975; Hurley et al., 1975) to study disseminated candidiasis in normal and compromised hosts. In normal guinea pigs the organs primarily infected are the kidneys and the heart (Hurley and Fauci, 1975). It was noted that the acute PMN inflammatory response of the host and a mononuclear leukocytosis occur four days after infection. In evaluating the effects of long and short acting glucocorticosteroids on guinea pig susceptibility to infection it was found that long acting steroid treatment potentiates infection, increases mortality, and suppresses established parameters of CMI. Short acting steroid treatment lacked this actibity (Hurley et al., 1975).

Experimental studies on candidiasis performed in the Soviet Union have been reviewed by Kashkin (1974). Noteworthy among these studies are two experimental animal models. One model employs hyperimmune animals, which are highly susceptible to intravenous challenge, emphasizing the possible role of an immune complex-like syndrome resulting in death of the animal. The second animal model is one designed to establish pulmonary candidiasis. Pulmonary candidiasis has received little attention in the literature. Such a model should yield valuable host-parasite interaction data, especially in light of the increased incidence of pulmonary candidiasis among critically ill patients (Utz and Beachner, 1971).

The experimental animal models described above emphasize several aspects of systemic candidiasis. These include the observations that: *i*. intravenous injection of large numbers of yeast cells in normal and compromised hosts causes an acute and rapidly fatal fungal infection; *ii*. injected cells are rapidly cleared from the blood-stream, the majority of yeasts becoming localized in the liver, spleen and lungs, and to a lesser extent the kidneys; *iii*. as infection progresses only the kidneys' population of yeasts increase in number while other organ populations are eliminated; *iv*. the elimination phase is accompanied by an acute PMN inflammatory response which decreases allowing yeasts to overgrow kidney tissue only; *v*. this

process is accelerated in the compromised host. Based on these observations surprisingly little attention has been given to the fact that the liver, spleen and lungs eliminate the major protion of the challenging dose in normal and immunosuppressed hosts.

Rogers (1960) reviewed the literature concerning the mechanisms by which bacteria are cleared from the bloodstream. There are multiple host mechanisms which interact to rapidly and efficiently remove circulating microbes and destroy them. Destruction takes place primarily inside phagocytic cells. The reserve capacity of these systems is rarely exceeded, although significant portions of the defense systems may be compromised. Numerous experimental models indicate that the majority of intravenously injected bacteria cleared from the bloodstream are trapped in the liver, spleen, and lungs. These organs contain the largest reserve of cells comprising the fixed tissue macrophage arm of the MPS. They represent the major organs of the classically described reticuloendothelial system (aschoff, 1924). The liver is the organ most often involved in the clearance of bacteria. The largest component of the MPS resides within the livers' resident macrophage population, the Kupffer cells.

The liver is uniquely interposed along the venous circulatory system between the gastrointestinal tract and the heart. Venous blood collected by small mesenteric

veins, the right and left gastric veins, and the splenic vein passes into the large portal vein. The portal vein and hepatic artery enter the liver at the porta hepatis and become highly branched. Blood percolates from the portal system into liver venous sinusoids. Arterial blood mixes with venous blood at the sinusoidal level traveling toward the heart. Lymph is collected in small lymphatic ducts and carried away from the sinusoids. Blood percolates through the sinusoids, between liver parenchymal cell plates, and is collected in central veins. Blood collected in central veins passes into the hepatic vein. It is returned to the inferior vena cava, just below the diaphragm, and finally passes into the right atrium of the heart (Warwick and Williams, 1973).

All of the blood entering the liver is filtered through the sinusoids prior to being recollected and returned to the heart. The structure and function of the sinusoid and sinusoidal cells is the subject of a continuing debate in the literature. The debate began over a hundred years ago. Aterman (1964) gives a detailed review of the controversy centers on the biology of Kupffer cells which live inside the sinusoidal lumen (Benacerraf, 1964).

The current concept regarding the fundamental anatomical relationships found in the sinusoid are emphasized in studies by Mills and Franklin (1969), Motta and Proter (1974), Wisse (1970, 1972) and have been reviewed (Wisse

and Daems, 1970). Sinusoids represent a highly anastomosed, flexible canal system. The sinusoidal lumen is lined by a double layer of fenestrated endothelium. Beneath this endothelial net parenchymal cell microvilli project onto the space of Disse. Kupffer cells occupy a considerable portion of the sinusoidal lumen, occurring most often at sinusoidal junctions. The Kupffer cell body is covered by numerous folds and microvilli. It is anchored to the fenestrated endothelial net by numerous, fine, cytoplasmic, dendritic processes. Nuclei of endothelial cells are easily distinguished from those seen in Kupffer cells in both scanning electron microscopic (SEM) and transmission electron microscopic (TEM) studies (Motta and Porter, 1974; Wisse, 1972). Kupffer cells are situated within the sinusoidal lumen in a manner advantageous to their exposure to a considerable portion of the circulating blood. In terms of the livers' cellular content about 38% of the total number of liver cells are Kupffer cells. This number represents about half of the total phagocytic capacity of the MPS (Howard, 1970).

As pointed out by aterman (1964) the major controversy concerning sinusoids has been resolving the origin of Kupffer cells. Van Furth and his colleagues (1968, 1970a, 1970b) showed that a bone marrow stem cell, the promonocyte, becomes the peripheral blood monocyte. The blood monocyte

is moderately phagocytic and upon exposure to a target cell, or antigen, it rapidly develops into the highly phagocytic peripheral blood macrophage. The majority of workers believe that the blood monocyte leaves the circulatory system after several days and takes up permanent residence in the liver sinusoid becoming the Kupffer cell (van Furth et al., 1975). The Kupffer cell divides slowly giving rose tp new Kupffer cells. Upon exposure to a microbe they may initiate more rapid reproduction (North, 1970).

Kinsky et al. (1969) studied the extrahepatic origin of Kupffer cells. CBA X CBA T6T6 F-1 mice given 850 r whole body X-irradiation were repopulated with CBA T6T6 bone marrow and CBA lumph node cells. Two weeks later animals were given five daily injections of diethylstilbesterol and their macrophages isolated. It was shown karyotypically that dividing macrophages in the liver are donor derived bone marrow cells. It was concluded that liver Kupffer cell proliferation is due to extrahepatic repopulation of liver macrophages by blood monocytes. North (1970) studied the influx of blood monocytes into the livers of animals infected with *Listeria monocytogenes* and concluded that these cells take up residence in liver sinusoids. They are morphologically indistinguishable from Kupffer cells except for their autoradiographic labeling patterns. Critical work concerning the linear

origin of Kupffer cells has been reviewed (Howard, 1970). The linear origin of Kupffer cells may not be a tenable hypothesis.

Volkamn (1976) used parabiosed inbred female Lewis rats to investigate the blood monocyte origin of PEC and Kupffer cells. His results indicate that Kupffer cells are not derived from blood monocytes but represent a self sustaining population of resident macrophages. It is unknown whether this disparity in theorigin of Kupffer cells has any functional significance. It must be kept in mind when designing models evaluating hepatic clearance of microbes either *in vitro* or *in vivo*.

In terms of the pathogenesis of *C. albicans* infection Kupffer cells could be postulated to play an important role in host resistance to infection. Actual invasion of the human and experimental animal host in both salmonellosis (Carter, 1975; Carter and Collins, 1974, 1975; Giannela, 1975; Tahechi, 1975) and candidiasis (Eras et al., 1972; Myerowitz et al., 1976) results in the introduction of these microbes into the portal venous system. Experimental models applicable to gram negative infections might also be applicable to *C. albicans* infection. An experimental model used to evaluate the role of Kupffer cells in antimicrobial resistance is the isolated perfused liver. Manwaring and Coe (1916) applied perfusion methods to a study of the role of

antipneumococcal opsonin present in immune serum. A heat stable opsonin was found to function only in liver "capillaries". Manwaring and Fritschen (1923) extended these original perfusion studies and defined eight "Laws" of tissue affinity governing the tissue distribution of microorganisms.

Howard and Wardlaw (1958) applied these Laws to a study of the opsonic effect of normal human, rat, and mouse sera on Kupffer cell phagocytosis of *Escherichia coli*. It was found that both specific opsonic antibody, complement, and possibly properdin contribute to the ability of Kupffer cells to phagocytize and kill *E. coli*. Bonventre and Oxman (1965) compared the phagocytosis and killing of *Staphylococcus aureus* and *S. enteritidis* using the perfused rat liver. They found that the immunological status of the rat had no effect on the fate of *S. aureus*. *Salmonella enteritidis* was phagocytized at an increased rate in immune serum. Components of the immune serum enhanced the ability of Kupffer cells to destroy bacteria.

The perfused liver model has facilitated an evaluation of the physiological status of Kupffer cells. Kupffer cells are actively phagocytic *in vitro* oxidizing glucose and acetate by the hexose monophosphate shunt pathway (Asiddao et al., 1964; Pisano et al., 1968, 1970). This activity is sensitive to glycolytic inhibitors such as iodoacetic acid (Pisano et al., 1968, 1970). In addition

to containing various hydrolytic enzymes and myeloperoxidase Kupffer cells possess the metabolic michinery for the generation of oxygen-dependent anti-microbial systems which would contribute to phagolysosome associated intracellular killing systems (Baboir, 1978; Johnston et al., 1975; Karnovsky, 1975).

It is generally acknowledged that Kupffer cells account for most of the trapping, by phagocytosis, and killing, as a result of phagocytosis, capacity of hepatic tissue (Aschoff, 1924; Baine et al., 1974; Bonventre and Oxman, 1965; Howard, 1961; Howard and Wardlaw, 1958; Jeunet et al., 1967, 1968, 1969; Manwaring and Coe, 1919; Manwaring and Fritsche, 1923; Rogers, 1960). Moon et al. (1975) showed that the rate of hepatic trapping of *S. typhimurium* is the same in the presence or absence of humoral factors. The perfused liver model was shown to closely approximate *in vitro* the realities of bloodstream clearance *in vivo*. It also allowed these investigators to distinguish between the bacterial trapping and bactericidal functions of hepatic tissue. They found that Kupffer cells require humoral factors for optimal phagocytic killing and that the initial event in hepatic clearance is trapping of bacteria in liver sinusoids outside Kupffer cells. In a subsequent study it was found that the macrophage specific toxin crystalline silica did not influence the ability of hepatic tissue to trap significant

numbers of *S. typhimurium* but completely abolished the ability of Kupffer cells to phagocytize and kill the bacteria (Friedman and Moon, 1977).

Few studies have been concerned with the interaction of *C. albicans* and hepatic tissue. Kemp and Solotorovsky (1962) employing fluorescent antibody methods combined with histopathological techniques followed the time course of *C. albicans* infections in mice. A large accumulation of yeast cells in the liver was observed immediately after intravenous injection. Complete clearance from the liver, as measured by loss of viability, was effected by the second day of infection. Initially large numbers of yeasts phagocytized by "endothelial cells", were unable to form blastospores. In this study no mention is made of the exact time at which intracellular yeasts were observed. There seems to be some doubt as to the identity of the phagocytic cell. Clarification of these observations is critical to an understanding of hepatic-*Candida* interactions. This is especially true in light of the hypothesis that the acute PMN inflammatory response is responsible for the initial elimination of *C. albicans* (Louria et al., 1960). The *in vitro* study of the kinetics of phagocytosis and intracellular killing of *C. albicans* demonstrates that phagocytosis of yeasts by human PMN and BM proceeds at equal rates with equivalent numbers of yeasts being internalized. PMN kill a significantly

larger number of the intracellular yeasts in a short time period (Leijh et al., 1977). The PMN could function efficiently in liver tissue to eliminate *C. albicans*.

Owstrowsky (1896) made the observation that leukocytosis, lymphadnopathy, hepatomegaly and splenomegaly are not features of human systemic candidiasis, implying that the MPS is not an active participant in the host defense system (Stanley and Hurley, 1969). Stanley and Hurley (1969) found that normal mouse PEC, in the presence of autologous serum, are killed within 24 h after ingesting *C. albicans* or *C. tropicalis*. Both yeasts rapidly develop germ tubes inside the macrophage. Meister et al. (1977) found that whole *C. albicans* blastospores and cell wall glucan are able to induce hepatic granuloma formation in mouse liver. Coupled with the studies by Leijh et al. (1977) and Kemp and Solotorvosky (1962) this data cumulatively suggests that macrophages in the liver may be incapable of destroying *C. albicans*. Yeast cells persisting in hepatic tissue contribute ultimately to granuloma formation. The major host defense response to *C. albicans* in the liver would be PMN inflammation occurring subsequent to hepatic clearance. In contrast to this hypothesis Baine et al. (1974) stated that Kupffer cell clearance, enhanced by the presence of fresh autologous rabbit serum, is responsible for the ability of perfused rabbit liver to remove yeast cells from the bloodstream and from perfusion

medium. Taken together, these studies also suggest that hepatic clearance of *C. albicans* might not be analogous to bacterial clearance.

At the end of his disucssion on bloodstream clearance mechanisms Rogers (1960) states that "to date we know of no way to enhance intracellular destruction of microbes". If Kupffer cells are, or are not, involved in the hepatic clearance of *C. albicans* it might be possible to enhance their activity. In accomplishing this host resistance to infection might also be enhanced. In retrospect Rogers' (1960) statement was only premature. The current concept of the acquired antimicrobial immune response postulates that as a result of infection by a viable intracellular parasite the host develops *in vivo* and *in vitro* correlates of CMI, a population of sensitized lymphocytes that passively transfer immunity, and acquisition of nonspecific enhanced antimicrobial macrophage activity (Mackaness, 1964, 1967). This immune response depends on a population of sensitized lymphocytes and macrophages (Mackaness, 1969; Miki and Mackaness, 1964). The macrophage component of this model is capable of nonspecifically killing a wide range of pathogenic microbes which nonsensitized macrophages allow to grow (Mackaness and Blanden, 1967).

Acquired CMI has been demonstrated to play a role in resistance to a variety of organisms other than bacteria (Ruskin and Remmington, 1968). Gentry and Remmington

(1971) enhanced resistance to *Cryptococcus neoformans* by infecting mice with either *Toxoplasma gondii, Besnoitia jellisonii,* or *L. monocytogenes*. Mara and Balish (1974) sensitized mice with *L. monocytogenes* and found them resistant, in terms of *in vivo* and *in vitro* CMI parameters, to intravenous challenge with *C. albicans*. The duration of resistance was short lived and sensitization with *C. albicans* did not enhance resistance to *L. monocytogenes* challenge. In these studies the organisms which confer nonspecific resistance are, for the most part, intracellular macrophage parasites.

In examining the genus *Corynebacterium* for species related to mycobacteria in terms of their lymphoreticular stimulatory properties, Halpern et al. (1963) found that *C. parvum* is a potent macrophage stimulator. *Corynebacterium parvum*, and other related *Corynebacterium* species, are bacteria of uncertain taxonomic definition. The extensive work of Johnson and Cummins (1972) and Cummins and Johnson (1974) clearly place *C. parvum* in the genus *Propionibacterium*. These authors consider *C. parvum* to be *P. acnes* an anaerobic, propinic acid producing, normal skin flora microbe. Cummins and Johnson (1974) have indicated that the name *C. parvum* and *P. acnes* are synonomous. In order to maintain continuity with the immunological literature the name *C. parvum* will be retained.

As an immunopotentiator *C. parvum* elicits a surprising number of *in vivo* and *in vitro* responses. Intravenous injection of *C. parvum* into mice causes splenomegaly and hepatomegaly (Castro, 1974; Dimitrove et al., 1977), stimulates the MPS (Adlam and Scott, 1972; Christie and Bomford, 1975; Halpern et al., 1963; O'Neil et al., 1973; Scott, 1972; Watson and Sljivic, 1976; Wilkinson et al., 1973; Zola, 1975), generates cytotoxic macrophages *in vitro* (Ghaffar and Cullen, 1976), enhances antibody production (Howard et al., 1973), retards the growth of tumors (Castro, 1974; Rao et al., 1977; Scott, 1974, 1975; Tuttle and North, 1975; Woodruff et al., 1976), and enhances lymphocyte trapping (Frost and Lance, 1973). It directly stimulates T and B lymphocytes (Howard et al., 1973; Zola, 1975), and activates complement by the properdin pathway in normal human serum (McBride et al., 1975). Treatment with *C. parvum* inhibits the ability of sensitized spleen cells to respond to PHA *in vitro* (Scott, 1972), causes transient anemia in mice given intravenous injections of *C. parvum* (McBride et al., 1974), blocks GVH reactions retarding graft rejection (Castro, 1974; Howard et al., 1973), decreases contact sensitivity to picrylochloride (Asherson and Allwood, 1971), and decreases the ability of liver to metabolize drugs (Soyka et al., 1976). Intravenous *C. parvum* treatment also increases resistance to infections by Herpes Simplex virus

(Kirchner et al., 1977), *L. monocytogenes* (Baughn et al., 1977; Ruttenberg and Jensen, 1975; Swartzberg et al., 1975), *T. gondii* (Krahenbuhl et al., 1976; Swartzberg et al., 1975), *Plasmodium berghei* (Nussenzweig, 1967), *S. enteriididis* (Collins and Scott, 1974), *S. typhimurium* (Fauve and Hevin, 1974), and *S. aureus* (Sher et al., 1975). Phospholipid extracts of *C. parvum* are able to enhance resistance to *S. typhimurium* and *L. monocytogenes* at a level approximateing that achieved using whole *C. parvum* cells (Fauve and Hevin, 1974).

Corynebacterium parvum fails to enhance resistance to infection with *Asperigillus nidulans* in DBA-2J mice (Purnell, 1976). When *C. parvum* was given prior to or with *A. nidulans* challenge mortality is significantly increased. Sher et al. (1975) treated CDF1 mice with *C. parvum* and BCG. Both immunopotentiators afforded significant protection against lethal *C. albicans* challenge. The mice were also immunosuppressed with 300 mg/kg cyclophosphamide given four days prior to bacterial vaccination.

The immunopotentiating mechanism of action of *C. parvum* has recently received experimental attention. Dimitrove et al. (1977) studied the organ distribution of iodine-125-labeled *C. parvum* injected by various routes in mice. It was found that intravenous injection resulted in maximal hepatomegaly and splenomegaly. The bacteria were

rapidly removed from the bloodstream. They persisted for the longest period of time, one week, in the liver. Localized injection of *C. parvum* results in a regional lymph node response, primarily a proliferative response of small lymphocytes (O'Neill et al., 1973). Ogmundsdottir and Weir (1976) showed that *in vitro C. parvum* adheres to the surface of PEC. A HCl-lipid extract, when preincubated with PEC, inhibits the binding of whole *C. parvum* to PEC. Trypsinization of PEC has no effect on adherence obviating a possible role for macrophage associated antibody in binding *C. parvum*. Addition of the major sugars found in *C. parvum* cell walls to the incubation medium and removal of divalent ions from the medium inhibited binding.

Christie and Bomford studied the *in vitro* (Christie and Bomford, 1975) and *in vivo* (Bomford and Christie, 1975) mechanism of macrophage activation by *C. parvum*. Their studies show that *in vitro* and *in vivo C. parvum* stimulates macrophage both directly and through immunological mediation by T lymphocytes. Studies by Tuttle and North (1976a, 1976b) demonstrated that *C. parvum* treatment generates a subpopulation of tumor specific, short lived, replicating T lymphocytes which passively transfer specific resistance to syngeneic fibrosarcoma in mice.

These studies suggest that *in vivo C. parvum* derives immunopotentiating abilities from its interaction with lymphocytes and cells of the MPS. Subsequent interactions

of these activated or sensitized lymphocytes and macrophages result in the variety of *in vitro* and *in vivo* biological activities seen in *C. parvum* stimulated experimental animal models (Howard et al., 1973). This is only a working hypothesis based on a few experimental models. The sequence of events *in vivo* following *C. parvum* treatment which results in the expression of acquired CMI to infection is essentially unknown.

In addition to a search for potentiators of both specific and nonspecific antimicrobial host defense systems a considerable amount of study has been devoted to the evaluation of inhibitors of these systems. Three such inhibitors employed in this study are crystalline silica, iodoacetic acid (IAA), and phenylbutazone (PB).

Crystalline silica is a specific cytotoxin for cells of the MPS (Allison, 1970, 1971; Allison et al., 1966; Comvili and Perin, 1963; Friedman and Moon, 1977; Kessel et al., 1963; Marks and nagelschmidt, 1959; Van Loveren et al., 1977). Kassel et al. (1963) clearly demonstrated that PMN are not susceptible to the cytotoxic action of silica. Silica enhances susceptibility of experimental hosts to Friend virus (Larson et al., 1972), Coxsackie B-3 virus (Roger-Zisman and Allison, 1973), Herpes virus (Zisman et al., 1970), *Mycobacterium tuberculosis* (Vorwald and Delahunt, 1938; Vorwald et al., 1954) both *in vivo* and *in vitro* (Allison and Hart, 1968), *Trypanosoma cruzi*

(Kierszenbaum et al., 1974), and *S. typhimurium* (Friedman and Moon, 1977).

Nadler and Goldfischer (1970) demonstrated that ingestion of silica by macrophages results in release of lysosomal enzymes into the macrophages' cytoplasm. The macrophage essentially commits suicide. Rios and Simons (1972) observed that poly-2-vinylpyridine n-oxide (PVO) reverses the ability of silica to induce macrophage intracellular degranulation by combining with the lysosome membrane and stabilizing it. Allison (1967) reviewed the mode of toxic action of silica on macrophage lysosomes. It is thought that silica is converted rapidly into silicic acid. The hydroxyl groups of silicic acid form hydrogen bonds with lysosome cell membrane phospholipids and proteins disrupting the integrity of the membrane. This would explain the stabilizing effect of PVO, its' oxygen atoms form hydrogen bonds with the hydroxyl groups of silicic acid to block hydrogen bonding between silicic acid and lysosome membrane components.

Levy and Wheelock (1975) observed that intravenous silica injection rapidly depresses the ability of the MPS to clear colloidal carbon from the bloodstream of mice. It reduces the *in vitro* phagocytic ability of PEC harvested three days after silica injection. Friedman and Moon (1977) found a slight depression in the carbon clearance ability of silica treated mice. In this study profound

effects were observed in the ability of silica compromised mice to clear intravenously injected *S. typhimurium* and to effectively kill the bacteria *in vivo* and *in vitro*. *In vitro* evaluation of the perfused mouse liver showed that silica treatment kills Kupffer cells without altering other anatomical features of the liver. The ability of the liver to trap perfused bacteria is reduced in these livers as is the rate of bacterial killing.

Iodoacetic acid (IAA), $C_2H_3IO_2$, prevents glycolysis by inhibiting phosphoglyceraldehyde dehydrogenase. It alters function of this enzyme by alkylation of cysteine 149 at the enzymes' active site. IAA also splits ribonuclease into two inactive fragments at pH = 5.5 (White et al., 1973). Assidao et al. (1964) found that 0.1 M IAA inhibited carbon clearance from perfusion medium in perfused rat liver. IAA was found to block oxidative Kupffer cell metabolism. Pisano et al. (1968) found that 3 mM IAA blocks phagocytosis of RE test lipid emulsion by Kupffer cells and 0.1 M IAA inhibited Kupffer cell hexose monophosphate shunt utilization of glucose (Pisano et al., 1970). Leijh et al. (1977) found that 1 mM IAA abrogated the ability of both PMN and BM to ingest *C. albicans*. The use of IAA to inhibit phagocytosis has been reviewed by Karnovsky et al. (1970). Taken together these studies indicate that IAA has an inhibitory effect on macrophages *in vitro* and *in vivo*.

Phenylbutazone (PB), $C_{19}H_{20}N_2O_2$, 4-Butyl-1,2-diphenyl-3,5-pyrazolidinedione, was found by Whitehouse (1964) to be an uncoupler of oxidative phosphorylation selectively inhibiting the biogenesis of ATP without blocking oxidative metabolism and cellular respiration. This metabolic inhibitor is used to effectively block the ability of phagocytic cells to kill intracellularly phagocytized microorganisms. Weissman (1966) found that high concentrations of PB prevented lysosomal enzymes from being released *in vivo* but it had no effect on isolated lysosomes *in vitro*. This observation appears to be incidental to the more profound effect of PB as a metabolic inhibitor in phagocytic cells.

Steigbigel et al. (1974) found that inclusion of 2 mg/ml PB in the incubation medium of either PMN or BM completely inhibited the ability of these cells to kill phagocytized *E. coli*, *L. monocytogenes*, *S. typhimurium*, and *S. aureus*. Solberg and his colleagues have studied the inhibitory effects of PB on PMN function. Killing of phagocytized *S. aureus* was inhibited by 2 mg/ml PB *in vitro* (Solberg, 1972). It was found that high concentrations of PB caused reduced phagocytic activity by PMN in addition to blocking intracellular killing of *S. aureus* (Solberg, 1974). At lower doses PB inhibited PMN CO_2 production by blocking the utilization of glucose in the hexose monophosphate shunt pathway inhibiting intracellular

killing. Strauss et al. (1968) studied the effects of varying concentrations of PB on the ability of guinea pig PMN to phagocytize and kill *E. coli*. Bactericidal PMN homogenates are also inhibited by PB *in vitro*. Metabolic evaluation of the effects of PB and PMN showed that the drug inhibits ^{14}C-glucose and ^{14}C-formate oxidation indicating an inhibitory effect on the hexose monophosphate shunt preventing H_2O_2 generation. This study also showed that PB inhibits glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activity. Leijh et al. (1977) found that 1 mM PB inhibited the intracellular killing of *C. albicans* by PMN and BM. This study implied that 1 mM PB inhibits only intracellular killing and not phagocytosis. This observation is substantiated by the work of Kjosen et al. (1976). They found that low concentrations of PB inhibit intracellular killing of *S. aureus* by human PMN by selectively blocking the hexose monophosphate shunt and not the Embden Myerhoff glycolytic pathway. Lehrer (1975) also found that PB inhibited the ability of BM to kill ingested *C. albicans*. Taken together these studies show that silica, IAA, and PB, inhibitors of phagocytic function *in vivo* and *in vitro*, may be used as tools to manipulate the macrophage-microbe interaction.

MATERIALS AND METHODS

Animals

Sprague-Dawley-derived male rats weighing 300 to 400 g were purchased from Spartan Research Animals, Inc., Haslett, MI. Animals were maintained under standard laboratory conditions with Purina Laboratory Chow and water available *ad libitum*.

Microorganisms

The strain of *Candida albicans* used in this study was isolated from a case of candidal vaginitis at the Olin Health Center of Michigan State University. Identity was confirmed by fermentation of glucose and maltose but not sucrose or lactose, chlamydospore production on 1% Tween 80 corn meal agar, and germ tube formation in 2 h at 37 C. Stock cultures were maintained on Sabouraud dextrose agar (SDA) slants at room temperature. The inoculum was prepared from a fresh transfer incubated under continuous agitation at 37 C overnight in 100 ml of Trypticase soy broth (Difco), pH = 7.4, supplemented with 4% d-glucose (Baine et al., 1974). Cells were harvested and washed three times in sterile saline. Standardization of the

inoculum was by hemocytometer counts and pour plates of 10-fold dilutions in Sabouraud dextrose agar.

Chemicals

Sodium barbital injection, 65 mg/ml (W. A. Butler, Co., Columbus, Ohio) was stored by refrigeration and rats were anesthetized by intraperitoneal injection of 9.5 mg/ml of barbital in sterile saline.

Heparin, sodium salt (Sigma Chemical Co., St. Louis, MO), Grade II was filter sterilized in sterile saline using Falcon 0.22 micron filters, No. 7103 (Falcon Div. of Becton Dickinson & Co., Oxnard, CA), to a concentration of 20,000 USP JA units/ml and stored as stock at 4 C. A 1:10 dilution of stock was made in sterile saline and rats were given 2,000 units of heparin intravenously (i.v.) in the dorsal vein of the penis.

Phenylbutazone (Lot No. 127C-0083) and iodoacetic acid (as free acid, Lot No. 37C-0375) were both obtained from Sigma Chemical Co., Columbus, Ohio. Both drugs were used in 1 mM concentrations prepared in fresh Medium 199 (M 199, Gibco). Upon suspension of each drug the pH of the solution was adjusted to 7.3 with 1 N NaOH and the solution resterilized by filtration in Falcon 0.22 micron filters.

Corynebacterium parvum vaccine was obtained as a gift from Dr. Richard L. Tuttle, Burroughs Wellcome Co.,

Research Triangle Park, N.C. Two lots of vaccine were used throughout this study. Lots CA 528A and CA 580A were formalin killed suspensions supplied at a concentration of 7 mg dry weight of bacteria/ml with 0.01% thiomersal. Control studies using stock vaccine indicated that 350 µg of *C. parvum* killed 10^6 *C. albicans* within 30 min at 37 C. This was due to the preservative. Despite the fact that various blood fractions from *C. parvum*-treated rats were not toxic for *C. albicans*, all experiments were conducted with *C. parvum* vaccine washed free of toxic perservative. No variation in response between lots of vaccine was observed.

Dorentrup silica (DQ 12), particle size approximately 5 microns, was kindly supplied by Dr. Robert J. Moon, Department of Microbiology and Public Health, Michigan State University. All suspensions were autoclaved in powder form and suspended in sterile saline at a concentration of 100 mg/ml. Prior to i.v. injection, DQ 12 silica was exposed to ultrasonic vibration by a Sonifier Cell Desruptor Model W 1400 (Heat Systems-Ultrasonics Inc., Plainview L.I., N.Y.) at 75 watts for 1 min to suspend the silica. Two 5 mg injections were given i.b. 48 h apart. The last silica injection was given 24 h prior to experimentation.

Corynebacterium parvum treatment

Rats were given either 350 µg or 1.4 mg washed *C. parvum* i.v. Each dose was administered under light barbital anesthesia either two days or ten days prior to experimentation.

Complete blood counts (CBC) and differential white blood counts

Leukocyte CBC were performed using Becton Dickinson Unopettes for manual white blood cell enumeration. Cells were counted in an improbed Neubauer chamber. Differential white blood cell counts were determined at the appropriate times from air dried, peripheral blood smears, obtained by cardiac puncture, stained with Wright stain. Monocytes, polymorphonuclear leukocytes (PMN), and lymphocytes were expressed as a relative percentage of the 100 total cells counted.

In vivo clearance and tissue distribution of *C. albicans* in rats

To measure clearance of *C. albicans* from the bloodstream of normal rats approximately 10^6 or 10^8 yeast cells were injected i.v. into the dorsal vein of the penis of barbital anesthetized, heparinized rats. A needle was inserted into the heart, and 1 ml blood samples were withdrawn at 0, 2, 5, 10, 15, 20, and 30 min. Quantitative plate counts determined viability. The distribution

of yeast cells among liver, lungs, spleen, kidnesy, and peripheral blood was determined 30 min and 60 min after injection in normal and *C. parvum*-treated rats. Viability was determined by standard tissue homogenization and quantative plate count methods (Moon et al., 1974).

Rat surgery and *in vitro* liver perfusion

Rats were anesthetized with barbital, heparinized and bled by cardiac puncture. When appropriate, plasma was immediately separated from peripheral blood cells by a single centrifugation at 2,000 X g for 10 min in a Phillips Drucker L-780 combination centrifuge. In one set of experiments blood was collected in Becton Dickinson Vacutainers containing 10.5 mg disodium Edetate (EDTA, ethylene diamine tetraacetic acid). For all other experiments blood was separated in Becton Dickinson red top Vacutainers.

Procedures for liver perfusion have been described in detail by Moon et al. (1975) and Exton (1975). A full length midline incision was made, the abdominal walls retracted and the viscera displaced to one side exposing the portal vein. A ligature was placed under the inferior vena cava above the right renal vein. Two ligatures were placed under the portal vein, one above the splenic vein and one below it. The portal vein was held taut with a forceps and a small cut made in the wall of the vein below

the two ligatures. A sterile polyethylene cannula (Becton Dickinson Co., Rutherford, NJ, ID, 0.046 inch; OD, 0.066 inch or Becton Dickinson Intramedic Tubing, ID, 0.047 inch; OD, 0.067 inch) filled with slowly flowing Gibco M 199 was inserted into the lumen of the portal vein and tied into place by the two ligatures.

The cannula led to a three way valve (Becton Dickinson Co., Model No. MS 3033) one port of which contained a 1 ml syringe containing sterile M 199 and the second port of which was attached to two 50 ml glass syringes filled with prewarmed sterile M 199. Since the valve was open to prevent introduction of air bubbles into the portal vein, the inferior vena cava was cut below the renal vein allowing a slow flow of M 199 through the liver and preventing swelling. The tip of the cannula did not extend into the portal hepatis. The thorax was reflected and a ligature placed under the inferior vena cava distal to the hepatic vein. The right atrium was held taut with a forceps, cut, and the efferent polyethylene cannula (ID, 0.046 inch; OD, 0.066 inch) was inserted, pushed into the inferior vena cava, and secured with the ligature. The ligature on the inferior vena cava above the right renal vein was tied and the perfusion medium collected from the efferent cannula. In most cases the time elapsed from the initial incision to tying the last ligature on the inferior vena cava was between 5 and 10 min. The liver

was kept moist by bathing it with warm sterile M 199 and covering it with a sterile plastic petri dish.

The liver was washed free of blood cells with a sterile M 199 (ca. 50 ml). The flow rate was carefully adjusted to establish a constant flow through the liver and did not exceed suggested rates of flow for isolated perfused livers (Miller, 1973). Prior to perfusion with yeasts the effluent was treated for sterility and any liver having more than ten colony forming units (CFU) per ml of effluent was excluded from the experiment. At the end of each experiment sterility of the effluent was checked in a similar manner.

After washing the liver, the 1 ml syringe containing M 199 on the three way valve was exchanged for a 1 ml syringe containing 1 ml of sterile saline and either 10^6 or 10^8 CFU of *C. albicans*. A sterile 125 ml Erlenmyer flask with a foil cover was placed at the efferent cannula which was inserted through the foil. The yeast cells were slowly and steadily infused through the three way valve and followed immediately by perfusion of 100 ml of M 199 collected in 30 min. In some experiments perfusion medium was supplemented with either 10% whole rat blood or 5% rat plasma. For 60 min and 3 h perfusions the Erlenmyer flasks were changed at 30 min intervals and 100 ml of perfusion medium was collected in 30 min. The previous 100 ml was held on ice until ready for dilution plating.

For quantitative plate counts, the liver was disconnected from the perfusion apparatus, excised, and placed in a 100 ml graduated cylinder. The volume was adjusted to 100 ml with sterile saline, and the livers were homogenized in a Waring blender for 2 min. Quantitative Sabouraud dextrose agar pour plates were made using 10 fold serial dilutions of the control, *C. albicans* in saline, liver homogenate, and effluent. Plates were incubated 48 h at 37 C and the number of CFU was counted manually.

The percentage of untrapped yeasts was calculated by the formula:

$$N = \frac{\text{number of CFU recovered in effluent}}{\text{number of CFU infused}} \times 100$$

The difference between the percentage of CFU recovered in the effluent and the total infused (100%) suggests the percentage remaining in the liver. The percentage of viable yeasts remaining in the liver plus the percentage recovered in the effluent when subtracted from the percentage infused (100%) indicated the percentage killed.

Control experiments characterizing the isolated perfused rat liver using 10^6 and 10^8 CFU indicated that liver flow rates remained normal after the yeasts were infused. After perfusion for either 30 min, 60 min, or 3 h with sterile M 199, an additional 1 ml portion was

collected and tested for sterility. This sample consistently yielded less than 10 CFU indicating that the yeast cells trapped in the liver could not be removed by continuous perfusion.

Macrophage inhibition studies

Both phenylbutazone and iodoacetic acid were infused directly into isolated perfused livers of rats receiving 350 µg *C. parvum* two days prior to use. Both drugs were used in 1 mM concentrations prepared in sterile M 199. These concentrations are non-toxic to *C. albicans* (Leijh et al., 1977). Once infused with either drug, livers were washed for 15 min with sterile M 199 prior to infusion of *C. albicans*.

Rats were given 10 mg DQ 12 silica i.v. as described above. Rats were injected i.b. with 350 µg *C. parvum* 24 h after the first 5 mg silica injection and two days prior to experimentation. Results from the silica injected *C. parvum*-treated rats were compared to control rats receiving only *C. parvum*.

Scanning electron microscopy (SEM)

Isolated perfused livers from normal and *C. parvum*-treated rats, with or without infused *C. albicans* were prepared for SEM by the methods of O'Donnell and Hooper (1977), with the following modifications. After perfusion with 10^8 yeast cells, the upper reservoir of the perfusion

apparatus was filled with freshly prepared warm 2% glutaraldehyde (Eastman Kodak, Rochester, NY) in sterile 0.2 M sodium phosphate buffer (2% GA-PB) at pH = 7.4, and 100 ml was perfused in 30 min. Fixed livers were excised, cut into small blocks, and allowed to stand in 100 ml of fresh cold 2% GA-PB overnight. Blocks were dehydrated in sequential 30 min steps with 10, 20, 40, 70, 90, 95, and 100% ethanol. The tissue was held overnight at 4 C in a fresh change of 100% ethanol. The dehydrated blocks were cyrofractured in liquid nitrogen. Fractured tissue was placed in metal baskets under liquid nitrogen and dried in an Omar SPC 900/EX critical point dryer using CO_2 as the carrier gas. The specimens were coated with gold (200-300 A) using the EMS-41 Minicoater (Film Vac. Inc., Englewood, NJ) and viewed in either an AMR-900 SEM or an ISI Super Mini II SEM.

Statistics

Statistical analysis was performed by the White Rank order test (Wilcoxon and Wilcox, 1949) and all data was reported with its standard deviation.

RESULTS

Clearance and tissue distribution of intravenously injected *C. albicans* in normal rats

In vivo clearance of 10^6 and 10^8 CFU of *C. albicans* from the bloodstream of normal rats is shown in Figure 1. By 5 min over 90% of the intravenously injected yeasts were cleared. Between 5 and 30 min the percentage of viable yeasts remaining in circulation fell below 1%. One hundred percent of the injected cells was recovered from reticuloendothelial (RES) organs as viable yeasts, 30 min after injection, at both dose levels. Over 90% were accounted for in the liver and lungs (Table 1). Less than 10% of the yeasts were in the kidneys.

Sixty minutes after intravenous injection of 10^6 CFU into normal rats only 56% of the total number of yeast cells were recovered from the same RES organs, 44% being killed (Table 1). The viable yeast cell distributions at 60 min remained essentially the same for the lungs, spleen, kidneys and peripheral blood, as that for 30 min. The liver population fell from approximately 63% of 10^6 injected yeast cells recovered at 30 min to 23% of the

Figure 1. Percentage of viable yeasts in rat blood at various times after intravenous injection of either 10^6 or 10^8 CFU of *C. albicans* (average $\pm$ standard deviation). Calculations were made assuming a 20 ml blood volume. Each value represents at least six separate experimental determinations.

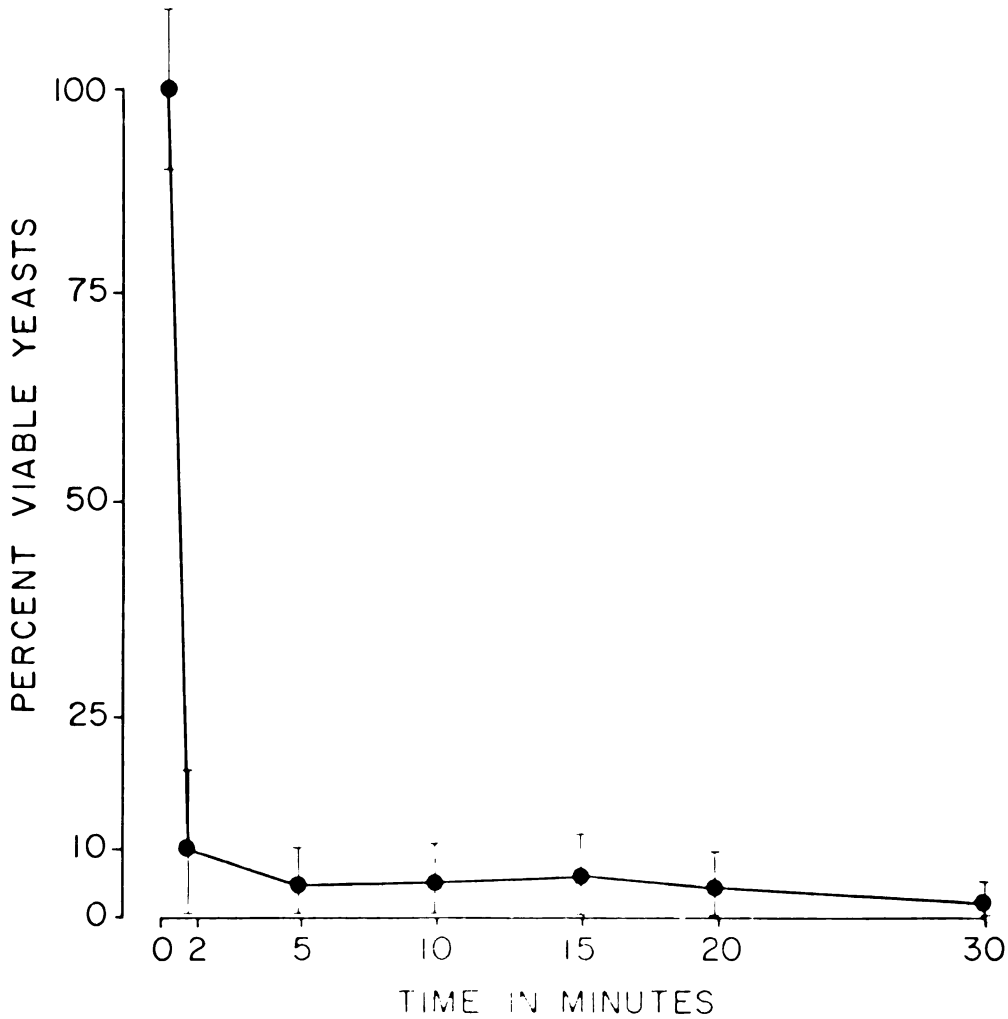

100
75
50
25
10
0
PERCENT VIABLE YEASTS
0 2
5
10
15
20
30
TIME IN MINUTES

Table 1. Survival of *C. albicans* 30 min and 60 min after intravenous injection into rats[a].

ORGAN	EXPERIMENTAL		
	30 min after 10^6 CFU injected i.v.	30 min after 10^8 CFU injected i.v.	60 min after 10^6 CFU injected i.v.
Liver	63 ± 10[b]	79 ± 4	23 ± 4
Lungs	27 ± 5	19 ± 2	27 ± 7
Spleen	4 ± 1	4 ± <1	2 ± <1
Kidneys	10 ± 2	2 ± <1	3 ± <1
Peripheral Blood	1 ± <1	1 ± <1	1 ± <1
TOTAL	105 ± 11	104 ± 5	56 ± 2
KILLING	0	0	44 ± 2

[a]Each value represents at least six separate experimental determinations.

[b]Percentage ± standard deviation.

same dose recovered 60 min after injection (P = 0.001).

Hepatic clearance of *C. albicans* by perfused livers from normal rats

The perfused rat liver cleared over 85% of 10^6 or 10^8 yeasts on a single pass (Table 2). In all but one instance (line 2, Table 2) all yeasts remained viable. The 15% killing found in this experimental group was not statistically significant. A similar experiment in the presence of blood did not significantly change trapping or killing (Table 3), even in perfusions lasting as long as three hours (Table 4), the total recovery of *C. albicans* being essentially 100%. There was no decrease in yeast cell viability when 10^6 CFU of *C. albicans* were incubated at 37 C, under gental agitation for 60 min, in the presence of normal rat plasma or normal rat blood (data not shown).

SEM of normal rat liver and *in vitro* clearance of *C. albicans* from the perfusion medium

Figure 2 shows micrographs of normal rat liver sinusoidal tissue. Figure 2A is a low magnification of cryofractured liver showing a portal vein with bifurcation. Small portal venules branch away from the portal vein toward central veins. Material in the portal vein is artifactual. Radiating from the portal venules are sinusoids which extend into a central vein. Higher

Table 2. Trapping of a single pass of viable *C. albicans* by the perfused rat liver after 30 min in the absence of blood[a].

No. of yeasts perfused	% RECOVERY			% Killing
	Effluent	Liver Homogenate	Total Recovery	
1.38×10^6	18 ± 12[b]	84 ± 13	102 ± 13	0
2.39×10^6	15 ± 3	70 ± 1	85 ± 2	15
1.49×10^8	14 ± 2	87 ± 13	101 ± 14	0

[a]Each value represents at least six separate experimental determinations.

[b]Percentage ± standard deviation.

Table 3. Trapping and killing of 10^6 *C. albicans* by perfused rat livers after 30 min in the presence of whole blood[a].

Experimental	% RECOVERY			% Killing
	Effluent	Liver Homogenate	Total Recovery	
No additives Medium 199 only	18 ± 2[b]	84 ± 13	102 ± 13	0
Medium 199 + 10% whole rat blood	15 ± 7	104 ± 17	119 ± 17	0

[a] Each value represents at least six separate experimental determinations.

[b] Percentage ± standard deviation.

Table 4. Trapping and killing of 10^6 *C. albicans* by perfused rat liver after 30 min, 60 min or 3 h continuous perfusion in the absence and presence of whole blood plasma[a].

Experimental	% RECOVERY			% Killing
	Effluent	Liver Homogenate	Total Recovery	
30 MIN PERFUSION				
M 199 only	4 ± 2[b]	90 ± 7	94 ± 8	6 ± 5[c]
M 199 + 5% plasma	6 ± 4	96 ± 3	102 ± 7	0
60 MIN PERFUSION				
M 199 only	4 ± 1	91 ± 3	95 ± 4	5 ± 2
M 199 + 5% plasma	9 ± 6	90 ± 10	99 ± 5	0
3 H PERFUSION				
M 199 only	7 ± 3[d]	103 ± 10	110 ± 14	0
M 199 + 10% whole rat blood	20 ± 7	85 ± 20	105 ± 17	0

[a] Each value represents at least five separate experimental determinations.

[b] Percentage ± standard deviation.

[c] $P = 0.50$

[d] Average of four 100 ml fractions collected over 3 h, the last three fractions contained less than 10 CFU/fraction.

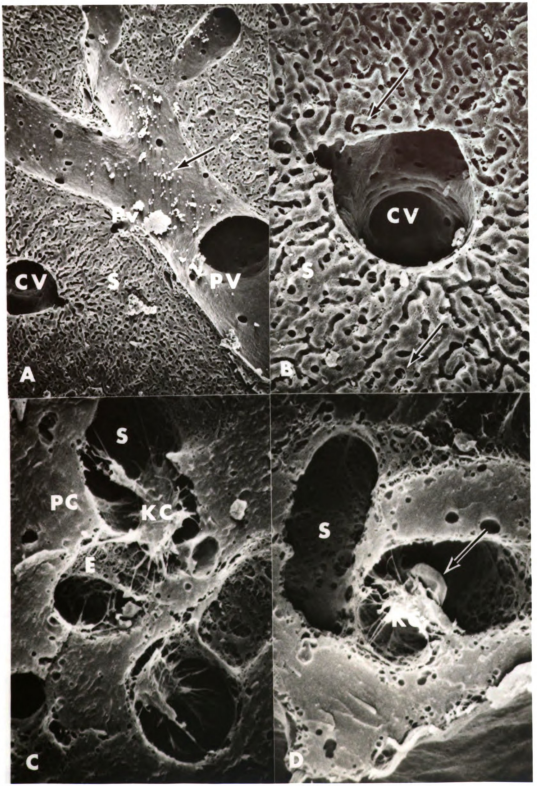
Figure 2. SEMs of normal rat liver

A. Cryofractured rat liver with branching portal vein (PV), portal venules (Pv), sinusoids (S), and central vein (CV). Material in the portal vein is artifractual (arrow). X 90.

B. Central vein (CV) with sinusoids (S). Areas indicated by arrows are magnified in C. and D. X 210.

C. Sinusoidal area (S) with fenestrated endothelial lining (E), parenchymal cells (PC), and Kupffer cells (KC). X 2,100.

D. Sinusoidal area (S) with Kupffer cell (KC) and attached erythrocyte (arrow). X 2,100.


CV
S
PV
A
CV
S
B
S
PG
KC
E
C
S
D

magnification of this area is shown in Figure 2B. Figures 2C and 2D are higher magnifications of the sinusoidal areas indicated by arrows in Figure 2B. Figure 2C shows the liver sinusoid, the lumen of which is lined by a double layer of fenestrated endothelium. Beneath the endothelial net, parenchymal cell microvilli project into the Space of Disse (not shown). Two Kupffer cells are attached to the endothelial cell cytoplasmic net by fine cytoplasmic dendritic processes. The surface of Kupferr cells had numerous folds and small villous projections. Figure 2D shows a Kupffer cell with an attached erythrocyte.

Figure 3 shows low magnification micrographs (380-760 X) of yeasts trapped in the liver following perfusion. Figure 3A is a micrograph of sinusoids packed with numerous yeast cells. Figure 3B shows a venule of the portal system also filled with yeasts. Figure 3C is a micrograph of a branch of the portal vein filled with yeasts. Figures 4A and 4B are higher magnifications of the sinusoidal areas containing *C. albicans*. Both figures show that yeast cells fill a considerable portion of the sinusoidal lumen. These figures also show that trapped yeasts were most often not associated with Kupffer cells.

Frequently pseudohyphae were observed. In Figure 5, venous sinusoids in the lower right of the micrograph contain *C. albicans*. The branches of the portal vein are also filled with yeasts. The inset in Figure 5 is a

Figure 3. Low-magnification SEMs of *C. albicans* trapped by the perfused rat liver.

A. Liver sinusoids (S) with numerous trapped *C. albicans* (arrows). X 380.

B. Portal venule (Pv) and sinusoids (S) containing trapped *C. albicans*. X 760.

C. Arteriole (A) and branch of the portal vein (PV) containing trapped *C. albicans*. X 760.

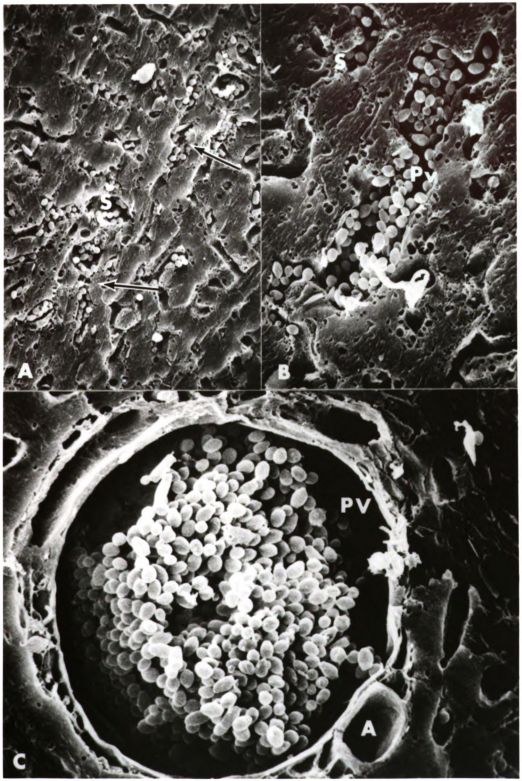

A
S
B
S
P
C
PV
A

Figure 4. High-magnification SEMs of *C. albicans* trapped in liver sinusoids.

A. *C. albicans* trapped inside liver sinusoids (S) partially filling the lumen of the sinusoid. X 2,150.

B. Kupffer cell (KC) and *C. albicans* trapped inside a liver sinusoid. X 2,200.

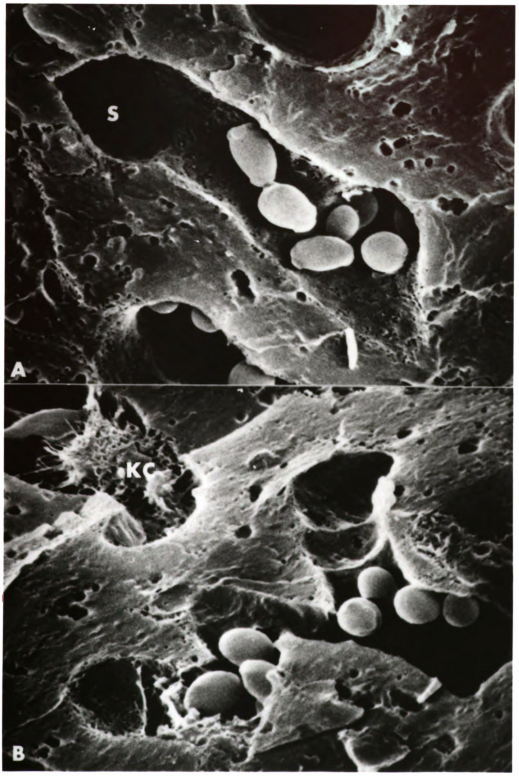
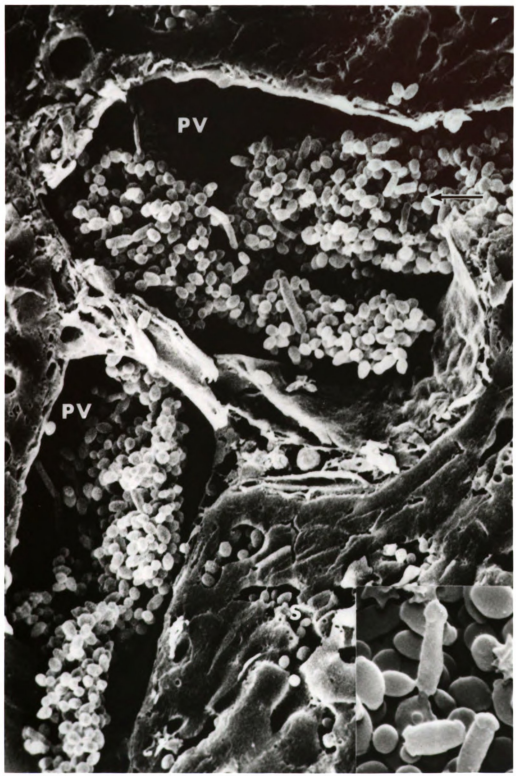

S
A
KC
B

Figure 5. SEM of trapped *C. albicans* showing pseudohyphae (arrow), liver sinusoids (S), and branching portal vein (PV) with trapped *C. albicans*. X 380. (Inset) Higher magnification of *C. albicans* with pseudohyphae (area indicated by arrow). X 3,800.


PV
PV
S

higher magnification of the area indicated by the arrow showing pseudohyphae. The pseudohyphae observed in these sections do not represent new elements formed during the short perfusion time since they were also observed in the inoculum.

Clearance and tissue distribution of intravenously injected *C. albicans* in *C. parvum*-treated rats.

Thirty minutes after intravenous inje-tion of 10^6 CFU of *C. albicans* into *C. parvum*-treated rats approximately 1% of the total inoculum was recovered from the peripheral blood (Table 5). Sixty minutes after injection, peripheral blood contained less than 1% of the total number of injected CFU. A total of 65% of the inoculum was recovered from various RES organs, with 26% in the liver 30 min after injection. The total recovery 60 min after injection was 56% with 27% in the liver. Sixty minutes after injection approximately 44% of the yeast cells were killed by *C. parvum*-treated rats.

Hepatic clearance of *C. albicans* by perfused rat livers from *C. parvum*-treated rats.

When *C. albicans* was infused into *C. parvum*-treated rat livers 95% of the yeasts were recovered after 30 min in the absence of plasma. Extending the perfusion time in *C. parvum*-treated rat livers to 60 min decreased the percentage of recovery to 78%, with 5% in the effluent

Table 5. Survival of 10^6 *C. albicans* 30 min and 60 min after injection into *C. parvum*-treated rats.

ORGAN	EXPERIMENTAL	
	Distribution 30 min after l.v. injection	Distribution 60 min after i.v. injection
Liver	26 ± 8[b]	27 ± 2
Lungs	32 ± 8	23 ± 5
Spleen	2 ± 0	3 ± 1
Kidneys	4 ± 1	3 ± 1
Peripheral Blood	1	0
TOTAL	65 ± 8	56 ± 4
KILLING	35 ± 6	44 ± 4

[a]Each value represents at least five separate experimental determinations.

[b]Percentage ± standard deviation.

and 73% in the liver. Addition of 5% plasma to the perfusion medium decreased the total percent of recovery to 66% with approximately 1% in the effluent and 64% in the liver (Table 6). There was no decrease in yeast cell viability when 10^6 CFU of *C. albicans* was incubated at 37 C, under gentle agitation for 60 min, in the presence of plasma or blood from *C. parvum*-treated rats (data not shown).

Dose and time relationship towards hepatic clearance of *C. albicans* by perfused livers from *C. parvum*-treated rats.

When rats receiving 350 µg *C. parvum* were perfused ten days after injection there was no increase in the killing of infused *C. albicans* in the absence or presence of plasma (Table 7). Treating rats with 1.5 mg *C. parvum* either two days or ten days prior to perfusion experiments in the absence or presence of plasma, did not significantly increase the ability of hepatic tissue to trap and kill *C. albicans* (Table 7).

White blood cell kinetics in normal, *C. parvum* and silica injected *C. parvum*-treated rats.

The CBC for normal rats was approximately 10,092 cells/mm^3, 5% of which were monocytes, 14% PMN, and 81% lymphocytes (Table 8). Forty eight hours after injection of 350 µg *C. parvum* the CBC increased to ca. 16,819 cells/mm^3 with the monocyte count elevated to 19% of the

Table 6. Trapping and killing of 10^6 *C. albicans* by perfused rat livers from *C. parvum*-treated rats in the absence and presence of rat plasma[a].

Experimental	% RECOVERY			% Killing
	Effluent	Liver Homogenate	Total Recovery	
30 MIN PERFUSION[b]				
M 199 only	3 ± 1[c]	92 ± 7	95 ± 6	5 ± 2
60 MIN PERFUSION				
M 199 only	5 ± 2	73 ± 2	78 ± 5	22 ± 5[d]
M 199 + 5% plasma	1 ± <1	64 ± 6	66 ± 6	34 ± 5

[a] Each value represents at least five separate experimental determinations.

[b] Injection of 350 µg *C. parvum* i.v. two days prior to experimentation.

[c] Percentage ± standard deviation.

[d] $P = 0.001$

Table 7. Trapping and killing of 10^6 *C. albicans* by perfused rat livers from *C. parvum*-treated rats in the absence and presence of rat plasma[a].

Experimental (*C. parvum* dose/time of exposure)	% RECOVERY			% Killing
	Effluent	Liver Homogenate	Total Recovery	
350 µg/ten days				
M 199 only	4 ± 1[b]	87 ± 5	91 ± 5	9 ± 5
M 199 + 5% plasma	1 ± 0	85 ± 2	86 ± 2	14 ± 1
1.4 mg/two days				
M 199 only	2 ± 1	84 ± 6	86 ± 6	14 ± 6
M 199 + 5% plasma	4 ± 1	68 ± 5	73 ± 6	27 ± 6
1.4 mg/ten days				
M 199 only	5 ± 2	82 ± 5	87 ± 4	13 ± 3
M 199 + 5% plasma	2 ± 1	81 ± 5	83 ± 6	17 ± 6

[a] Each value represents at least five separate experimental determinations.

[b] Percentage ± standard deviation.

differential count. Ten days after injection, the CBC and percentage of monocytes remained elevated.

The CBC of rats treated with 1.4 mg *C. parvum* decreased to ca. 6,000 cells/mm^3 two days after treatment but was statistically elevated by ten days after treatment. At both times the monocytes were elevated relative to the CBC. In both normal and *C. parvum*-treated rats the relative percentage of lymphocytes remained unchanged. The relative percentage of PMN decreased slightly in rats treated with 350 µg *C. parvum*.

In *C. parvum*-treated rats given silica the CBC was ca. 8,688 cells/mm^3 and the relative differential cell count was unchanged compared to normal rats (Table 8).

Inhibition of phagocytosis and phagocytic killing of *C. albicans* by perfused livers from *C. parvum*-treated rats.

In the presence of 5% plasma perfused livers from *C. parvum*-treated rats killed approximately 40% of 10^6 infused *C. albicans* after 60 min (Table 9). *Corynebacterium parvum*-treated rat livers exposed to either 1 mM phenylbutazone (PB) or 1 mM iodoacetic acid (IAA) were unable to kill hepatically trapped *C. albicans* in the presence of 5% plasma (Table 9). Trapping ability was not effected by treatment of livers with either drug since the percent recovery in the effluent was not significantly different from controls. Collection of whole rat blood

Table 8. White blood cell kinetics in normal, *C. parvum* and *C. parvum*-treated rats injected with silica[a].

Experimental (*C. parvum* dose/time of exposure)	CBC cells/mm^3	RELATIVE %		
		Monocytes	PMN	Lymphocytes
Normal rats	10,092 ± 320[b]	5 ± 1	14 ± 2	81 ± 2
350 µg/two days	16,819 ± 6945	19 ± 3	6 ± 3	75 ± 3
350 µg/ten days	11,913 ± 1593[c]	18 ± 3	7 ± 2	75 ± 6
	11,013 ± 3371	19 ± 4	5 ± 2	76 ± 4
1.4 mg/two days	6,644 ± 276	15 ± 4	9 ± 4	76 ± 8
	5,088 ± 3028	14 ± 3	7 ± 4	79 ± 6
	7,783 ± 3322	9 ± 5	11 ± 5	80 ± 6
1.4 mg/ten days	16,472 ± 3000	11 ± 6	9 ± 2	80 ± 8
	12,793 ± 2601	13 ± 6	11 ± 7	76 ± 6
350 µg/two days 10 mg DQ 12 silica	8,688 ± 3600[d]	5 ± 2	18 ± 4	77 ± 2

[a]Each value represents at least six separate experimental determinations.

[b]Percentage ± standard deviation.

[c]Separate experimental groups of at least six animals per group.

[d]Experimental group of ten animals.

Table 9. Inhibition of phagocytosis and phagocytic killing of 10^6 *C. albicans* by perfused rat livers from *C. parvum*-treated rats[a].

Experimental (*C. parvum* dose/60 min perfusion ± 5% plasma)	% RECOVERY			% Killing
	Effluent	Liver Homogenate	Total Recovery	
350 µg *C. parvum* M 199 + 5% plasma	2 ± 1[b]	59 ± 8	61 ± 8	39 ± 8
350 µg *C. parvum*[c]				
M 199 + 5% plasma + 1 mM PB	7 ± 3	93 ± 10	100 ± 7	0
M 199 + 5% plasma + 1 mM IAA	2 ± 1	111 ± 12	113 ± 12	0
M 199 + 5% plasma + 10.5 mg EDTA	3 ± 1	99 ± 9	102 ± 2	0
350 µg *C. parvum* 10 mg DQ 12 silica				
M 199 only	17 ± 10	88 ± 7	105 ± 4	0
M 199 + 5% plasma	19 ± 10	83 + 2	102 + 5	0

[a] Each value represents at least five separate experimental determinations.

[b] Percentage ± standard deviation

[c] Additives to perfusion medium were PB (phenylbutazone), IAA (iodoacetic acid), and EDTA (ethylene diamine tetraacetic acid).

in the presence of 10.5 mg EDTA abrogated the ability of plasma to enhance *in vitro* killing of *C. albicans* by perfused rat livers from *C. parvum*-treated rats (Table 9). Five percent of the infused yeasts were recovered in the effluent and 99% were recovered in the liver homogenate after 60 min.

Silica treated *C. parvum* rat livers, in the absence of plasma, were not as efficient in trapping or killing yeasts as were livers from normal rats (Table 4) or rats treated with *C. parvum* alone (line 1, Table 9). Seventeen percent of the infused yeasts were recovered in the effluent and 88% in the liver homogenate. Addition of plasma to the perfusion medium did not significantly alter this distribution. In the absence or presence of plasma silica treatment decreased the trapping ability, and abolished killing of *C. albicans* by *C. parvum*-treated hepatic tissue.

SEM of *C. parvum*-treated rat liver.

SEM studies of *C. parvum*-treated rat livers revealed morphologically dissimilarity compared to normal livers. Forty eight hours after treatment with *C. parvum*, a branch of the portal vein had numerous blood cells adhering to the wall (Figure 6A and 6B). Several cells morphologically similar to lymphocytes with round cell bodies and numerous villi can be seen. In addition two

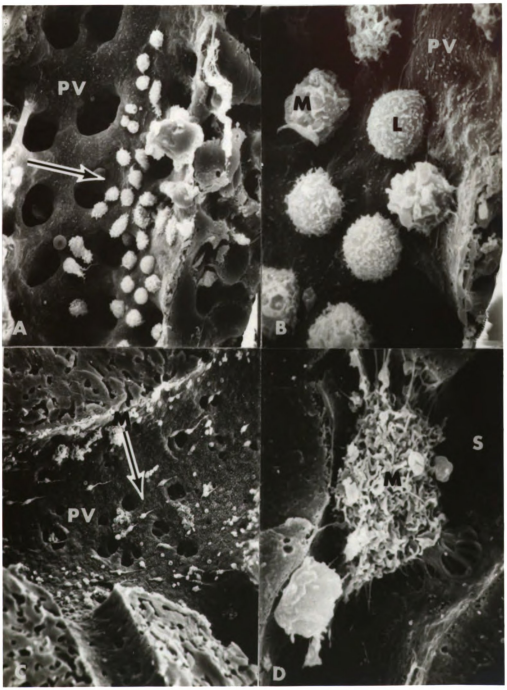
Figure 6. SEMs of rat livers two days after injection of 350 µg *C. parvum*.

A. Portal vein (PV) with adhering white blood cells (arrow). X 700.

B. White blood cells which adhere to portal vein walls (PV) in A, showing lymphocytes (L) and macrophages (M). X 3,000.

C. Portal vein with branches (PV) and adhering macrophage (M) with tail (arrow). X 200.

D. Higher magnification of a macrophage (M) in a sinusoid (S). The macrophage surface has numerous folds and projections and is attached to the endothelium by cytoplasmic processes. X 3,000.


PV
A
PV
M
L
B
PV
C
S
M
D

cells believed to be phagocytic cells of unknown identity can be observed. Figure 6C shows another portal vein with bifurcation. Macrophages observed adhering to the vein wall displayed typical "tails" which extended downstream or away from the "head" of the macrophage which was upstream or against the flow of blood. Figure 6D shows a sinusoid almost completely occluded by a macrophage. The cytoplasmic dendritic processes extend away from the macrophage body anchoring it to the endothelial lining of the sinusoid.

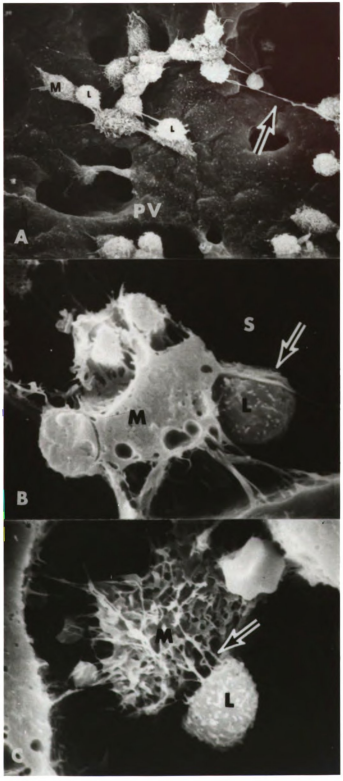
Figure 7A is a higher magnification of a portal vein with white cells adhering to the vein wall. There was a close association between lymphocytes and macrophages adhering to vein walls. Many fine cytoplasmic filaments extend between adjacent cells. Figure 7B and 7C show high magnifications of liver sinusoids. Figure 7B shows a macrophage and several attached lymphocytes. Numerous cytoplasmic filaments extend from the macrophage surface trapping the lymphocytes. Figure 7C shows a macrophage adhering to the endothelium of a sinusoid and attached to, or interacting with, a lymphocyte. Fine cytoplasmic appendages extending away from the folded surface of the macrophage contact the surface of the lymphocyte.

Figure 7. High magnification SEM of cellular interactions in *C. parvum*-treated rat livers.

A. Portal vein (PV) with clusters of macrophages (M), lymphocytes (L) and cytoplasmic filaments (arrow) attached to the portal vein wall. X 700.

B. Macrophage (M) in a sinusoid (S) with attached lymphocytes (L) which are surrounded by cytoplasmic filaments (arrow). X 5,000.

C. Macrophage (M) in a sinusoid (S) and a lymphocyte (L) attached by several cytoplasmic appendages (arrow). X 4,000.


M
L
L
PV
A
S
M
L
B
M
L

SEM of *C. albicans* trapped in perfused livers of *C. parvum*-treated rats.

Figures 8 through 10 show micrographs of livers from *C. parvum*-treated rats following perfusion with *C. albicans*. Figure 8 shows trapping of yeast cells along the walls of portal veins of decreasing diameter. Figure 8A shows massive accumulation of white cells and yeast cells in clusters in a large portal vein. Figure 8B shows a smaller portal vein, its' branches, and adjacent sinusoids, all of which were filled with adhering white cells and yeast clusters. Figure 8B shows a portal venule which was clogged with white cells and yeast clusters.

A higher magnification of yeast clustering is shown in Figure 9. Yeast clusters consist of numerous tightly adhering yeast cells, blastospores, and occasionally pseudohyphae. The yeast clusters adhered to the walls of portal veins. Many fine cytoplasmic filaments entangled the clusters. Pseudohyphae were considerably larger than the cytoplasmic filaments (Figure 9A). Yeast clusters were often associated with, or surrounded by, several macrophages. The macrophages were either attached to the portal vein wall or in the process of phagocytizing the yeasts. Cytoplasmic filaments interconnected macrophages and yeast clusters (Figure 9B). Yeasts, with and without attached filaments, were log jammed in portal venules prior to entering sinusoidal areas

Figure 8. SEM of *C. albicans* clearance in portal veins of perfused livers from *C. parvum*-treated rats.

A. Portal vein (PV), and sinusoids (S), filled with white blood cells and yeast clusters. X 100.

B. Portal vein branches (PV), and sinusoids (S), filled with white blood cells and yeasts. X 100.

C. Portal venule (Pv) with adjacent sinusoids (S) both containing clusters of white blood cells and yeasts. X 200.

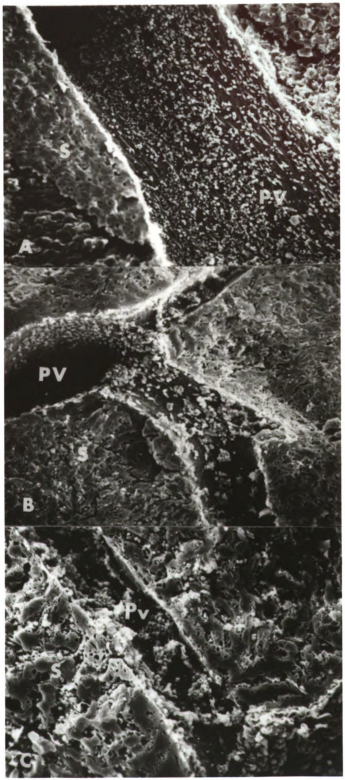

S
PV
A
PV
S
B
Pv
S
C

Figure 9. SEM of *C. albicans* trapping by perfused livers from *C. parvum*-treated rats.

A. Yeast cluster with pseudohyphae (arrow) and attached cytoplasmic filaments (F arrow). X 1,000.

B. Yeast cluster with attached macrophages (M) and cytoplasmic filaments (arrow). X 1,000.

C. Portal venule (Pv) with log jammed *C. albicans* which fills the adjacent sinusoids (S). Cytoplasmic filaments are attached to several yeasts (arrow). X 700.

D. *C. albicans* (Ca) in a sinusoid (S) attached to and in proximity to a macrophage (M). X 2,800.

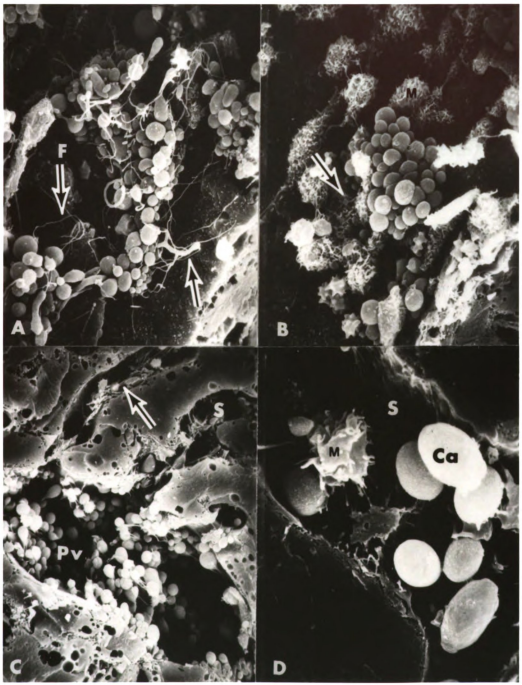
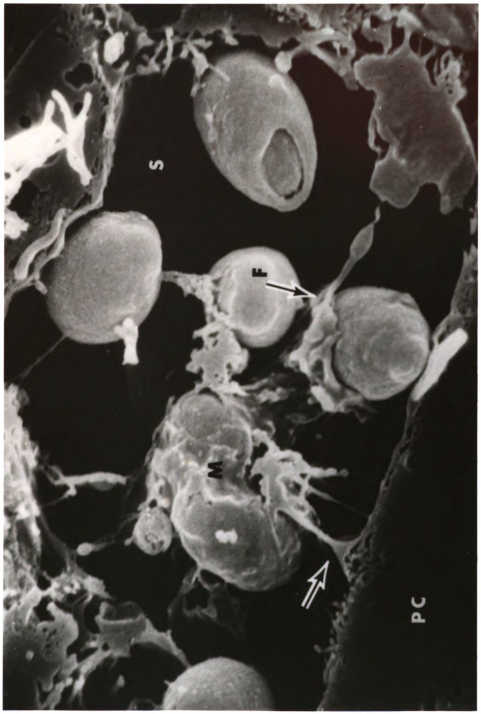

F
A
M
B
S
Pv
C
S
M
Ca
D

Figure 10. SEM of macrophage phagocytosis of *C. albicans* in perfused rat liver from *C. parvum*-treated rats showing a parenchymal cell (PC) and a sinusoid (S) with a macrophage (M) attached by cytoplasmic processes (arrow) to the endothelium. The macrophage has extended a cytoplasmic filament around a yeast cell (F arrow) pinning it to the endothelial lining of the sinusoid. X 6,000.


S
E
M
PC

(Figure 9C). *Candida albicans* was also trapped inside liver sinusoids near several macrophages occluding the sinusoid.

Figure 10 is a high magnification of a macrophage, located in a sinusoid, in the process of engulfing several *C. albicans*. The macrophage was attached to the endothelium by its cytoplasmic processes. The macrophage extended a cytoplasmic filament around one of the yeast cells, pinning it against the endothelial lining of the sinusoid, engulfing the yeast.

DISCUSSION

The ability of the liver to clear and kill *C. albicans* is evident in clinical literature. Eras et al. (o972) found that in human candidiasis the route of infection is frequently penetration of the gastrointestinal epithelium with subsequent hematogenous dissemination. Myerowitz et al. (1977) observed that gastrointestinal infection preceeds systemic invasion in cases of disseminated candidiasis. They found that 75% of their patients with proven disseminated candidiasis had hepatic in addition to kidney involvement. Louria et al. (1962) found primarily kidney involvement in their study. The mechanism by which *C. albicans* is eliminated from the liver, and the mechanism of its initial clearance in this organ, is poorly understood.

It might be postulated that during human and experimental candidiasis the liver serves as a site where host defense mechanisms would restrict further hematogenous dissemination from the gastrointestinal tract. Useful information might be obtained from an *in vitro* model which could be experimentally manipulated and which

approximates the initial bloodstream clearance of *C. albicans*. Moon et al. (1975) demonstrated, with respect to vascular clearance of *Salmonella typhimurium*, that the isolated perfused liver is an experimental model which approximates *in vitro* the *in vivo* clearance and killing of bacteria. The perfused rat liver not only reflects the ability of the host to kill hepatically trapped *S. typhimurium*, but the model clearly defines a new functional role for the liver, that of microbial trapping in liver sinusoids. Phase contrast and transmission electron microscopy of sinusoidal areas showed bacteria "log jammed" in sinusoids and outside liver Kupffer cells. Trapped bacteria are killed by Kupffer cells when humoral factors are added to perfusion medium

In contrast to the bacterial model, when a single pass of *C. albicans* was infused into normal rat livers there was no significant killing of the yeasts in the absence or presence of humoral factors (Table 2 and 3). At both doses studied yeast cells were cleared with equal efficiency (Figure 1, Table 2 and 3). When the perfusion medium consisted of M 199 plus 10% rat blood there was no change in the ability of the perfused rat liver to clear and kill *C. albicans* (Table 3). In fact total recovery in the absence or presence of whole blood or plasma exceeded 1000%, even after three hours (Table 4).

Baine et al. (1974) showed that *C. albicans* was rapidly removed from the bloodstream of rabbits. Distribution of yeast cells among various RES organs was dependent on the route of intravenous injection. Iannini et al. (1977) also studied the vascular clearance of *C. albicans* in rabbits comparing clearance of yeast cells to pseudohyphae clearance and found that the liver is extremely efficient to the clearance of both, *In vivo* clearance (Figure 1) and tissue distribution data (Table 1) confirm that in rats the majority of injected yeast cells were removed by the liver within 30 min. After 60 min the distribution of yeast cells had not significantly changed with respect to the lungs, spleen, kidneys, and peripheral blood. The percent recovery in the liver decreased 40%. Hence, there is general agreement with the literature that the liver and lungs are the major organs involved in bloodstream clearance of *C. albicans* (Baine et al., 1974; Hurley, 1966; Hurley and Fauci, 1975; Iannini et al., 1977; Kemp and Solotorovsky, 1962), and that subsequent to hepatic clearance yeast cells are rapidly killed.

In the study by Baine et al. (1974) it was found that *in vitro*, 90% of the yeasts were cleared from Krebs-Hanseleit buffered perfusion medium over a 60 min perfusion time. Addition of 5% heat inactivated normal rabbit serum to the perfusion medium increased clearance to 96%. In buffer with fresh 5% normal rabbit serum

clearance increased to 98%. The increase was found to be statistically significant. After examining the hepatically cleared yeasts by transmission electron microscopy it was concluded that clearance was promoted by heat stable and heat labile serum factors and that Kupffer cells accounted for the uptake of *C. albicans* by rabbit liver.

On closer analysis certain discrpancies between the rabbit and rat models become apparent. For example, in the rat model, addition of whole blood (Table 3 and 4) to the perfusion medium did not enhance trapping or killing, even after extended perfusion times. In the rabbit, the liver clears *C. albicans* more efficiently in the presence than in the absence of 5% rabbit serum. Viability alone was measured in our study while Baine et al. (1974) measured the percentage of recovery by a quantitative comparison of radioactively labeled yeasts with viability. In the rat no problem with aggregation of yeasts was encountered using M 199. Baine et al. (1974) employed a buffered perfusion medium containing bovine serum albumin (BSA) to reduce aggregation of yeasts. Aggregation is probably due to low levels of anti-*Candida* agglutinating antibody (Mathews and Inman, 1968) which may clump *C. albicans* in rabbit serum (Smith and Louria, 1972). Unpublished observations in the Medical Mycology laboratory of Dr. Everett S. Beneke, Michigan

State University, indicate that normal rat serum does not clump *C. albicans* when incubated at 37 C for up to 60 min.

Jeunet and Good (1969) studied the effects of soluble and heat aggregated BSA on the clearance ability of the perfused rat liver. Aggregated BSA produced reticulo-endothelial blockage due to exhaustion of plasma opsonin and stauration of Kupffer cells. A possible explanation of why rabbit liver clear 98% of infused yeasts in the presence of humoral factors while rat livers clear only 80% may be that a *C. albicans*-BSA interaction enhances trapping of yeasts. Whether differences between the rat and rabbit models reflect animal, experimental procedure, assay, or observational variations is not presently known.

When hepatic clearance of *C. albicans* is compared to hepatic clearance of bacteria, insights into the interaction of hepatic tissue with bacteria and fungi may be obtained. Howard and Wardlaw (1958), using the perfused rat liver, found that normal human, rat, and mouse sera were opsonic. These sera enhanced phagocytosis of *Escherichia coli* by Kupffer cells. The opsonic component of human serum was heat labile, suggesting a complement origin, and absorbable, implying the presence of *E. coli* specific opsonic antibodies. They suggest that opsonins were also generated from preperdin activation of

complement. Bonventre and Oxman (1965) used the perfused rat liver to evaluate the phagocytosis and killing of a gram positive and gram negative bacteria. They found that the immunological status of the rat had no effect on clearance and killing of *Staphylococcus aureus* by the perfused liver. Immune serum increased the rate and degree of phagocytosis of *S. enteritides* resulting in their complete destruction by the perfused rat liver. Moon et al. (1975) employed normal rat serum in their perfusion system. Addition of whole blood or plasma to the perfusion medium reduced the percent recovery in the effluent and in the liver resulting in approximately 55% of the infused bacteria being killed by the liver. This study made no attempt to characterize the plasma components in normal rat plasma which had such a profound effect on the fate of *S. typhimurium*. These studies suggest that humoral components from normal and immune rats enhance the ability of liver Kupffer cells to kill hepatically trapped bacteria.

Solomkin et al. (1978) showed that PMN phagocytic killing of *C. albicans* occurs optimally when yeasts are opsonized by specific opsonic antibody and complement opsonins, derived from either the classical or alternate pathways. Data presented in this study (Table 3 and 4) suggests that plasma factors do not enhance the ability of normal hepatic tissue to trap and kill *C. albicans*.

The data, when taken together with data obtained using bacterial systems, confirms Manwaring and Fritschen's (1923) "second law" of microbic-tissue affinity, namely "The microbic-tissue affinity varies with the micro-organism tested". A correlary to this "Law" might be postulated to state that tissue affinity varies also with respect to trapping and killing of the particular microbe being tested.

Moon et al. (1975) showed that *S. typhimurium* is initially log jammed in liver sinusoids extracellularly. Scanning electron microscopy (SEM) studies (Friedman and Moon, 1977) showed bacteria adhering to the sinusoidal endothelium. SEM studies were made to find out whether hepatic clearance of *C. albicans* in normal rats is a function of Kupffer cell or sinusoidal trapping.

Previous studies on the SEM of *C. albicans in vitro* (Joshi et al., 1973) and on the SEM of rat liver sinusoids (Motta and Porter, 1974) are consistent with the anatomical relationships demonstrated in this study. Light microscopic studies have indicated similar relationships to the SEM studies presented here (Hurley, 1966; Kemp and Solotorovsky, 1962; Louria et al., 1960, 1963). Extensive studies evaluating various tissue preparation methodologies show that perfusion-rapid fixation techniques including cryofracturing, and critical point drying are least likely to introduce artifacts in

host tissues (O'Donnell and Hooper, 1977; Weiss, 1972).

Transmission electron microscopic studies (Weiss, 1972) and SEM studies (Motta and Porter, 1973) are not particularly clear as to the nature of Kupffer cell orientation within the sinusoid. Kupffer cells have large bodies covered with many folds and microvilli which cover the body of the cell (Figure 2 and 4). The cell occupies a considerable portion of the lumen of the sinusoid, usually at the junction of anastomosing sinusoidal branches. Bloodstream borne microbes must come into intimate contact with the surface of Kupffer cells as they pass through the sinusoid. Extending away from the body of the cell fine, cytoplasmic dendritic processes anchor the cell to the fenestrated endothelial lining of the sinusoid. Cytoplasmic processes vary in length, shape and size and may be branched. They terminate, in almost all cases observed in this study, on the surface of the fenestrated endothelial cells. Termination of these processes on parenchymal cell microvilli was not seen in this study. Both transmission electron microscopy (Weiss, 1970, 1972) and SEM (Motta and Porter, 1973) suggest that they occasionally do.

When normal livers were infused with *C. albicans* small numbers of yeast cells became log jammed in sinusoidal spaces (Figure 3, 4, and 5). Yeasts appear to adhere to the endothelium of the sinusoid. It is envisaged that

trapping within the sinusoid restricts passage of more yeasts through the lumen. As yeasts filled the sinusoids they bagan to back up into portal venules and eventually became trapped in branches of the portal system. Individual yeast cells occupy a portion of the sinusoidal lumen making passage through the lumen restricted. It is not known whether trapping is a physical phenomenon or involves chemical interaction of yeast cell walls with sinusoidal endothelial cell membranes.

King et al. (1977) found that *C. albicans* readily adhere to vaginal epithelium although the mechanism of adherence was not examined. If physical trapping were the only trapping mechanism it might suggest that a smaller organism would pass through the liver and not be trapped. This is not the case. *Salmonella typhimurium* also adheres to sinusoidal endothelium (Friedman and Moon, 1977). Sterility controls taken from perfusion medium 30 min after infusion of yeasts contained fewer than 10 CFU/ml indicating that the yeasts were stuck in the liver.

In normal rat liver *C. albicans* trapping occurs without Kupffer cell involvement (Figure 4). Baine et al. (1974) show one transmission electron micrograph in which a yeast is being phagocytized. Our SEM study suggests that this may be an isolated event. There is no anatomical basis for their statement that the cell phagocytizing the yeast is, in fact, a Kupffer cell. The

majority of yeast cells seen in the SEM characterization were not associated with Kupffer cells. The inability to resolve the identity of Kupffer cells by either transmission electron or light microscopy shed light on the observation by Kemp and Solotorvsky (1962) that *C. albicans* was inside "endothelial phagocytes". It also points out the superior value of SEM to clearly identify Kupffer cells in sinusoidal areas of the liver.

These observations taken together characterize the initial trapping of *C. albicans* by normal rat hepatic tissue. It does not explain how the trapped yeasts are eliminated. If tissue distribution was examined 60 min after intravenous injection it was found that there was a significant decrease in the number of viable yeasts trapped in the liver (Table 1). How are these cells eliminated? This is an expecially acute question since extended perfusion times (Table 4), in the absence or presence of plasma, did not increase killing of *C. albicans*. Control studies indicated that whole rat blood is not candidacidal *in vitro*.

There is ample evidence that once *C. albicans* is trapped inside hepatic tissue a rapid PMN leukocytosis occurs (Baine et al., 1974). This response may be initiated either by the release of chemotactic factors from the yeast itself (Denning and Davies, 1973; Cutler,

1978) or by complement activation (Solomkin et al., 1978). Numerous *in vitro* studies have clearly demonstrated the candidacidal activity of the PMN (Arai et al., 1977; Davies and Denning, 1972; Denning and Davies, 1973; Glasser et al., 1977; Lehrer, 1970, 1972; Lehrer and Cline, 1969; Leijh et al., 1977; Schmid and Brune, 1974; Solomkin et al., 1978; Venkatraman et al., 1973; Yamamura et al., 1976; Zeya and Spitznagel, 1966). The sequence of events by which hepatically trapped *C. albicans* is eliminated *in vivo* in normal rats involves an initial trapping of yeasts in liver sinusoids followed by PMN inflammation (Hurley, 1966; Hurley et al., 1975; Louria et al., 1960, 1963; Kemp and Solotorvsky, 1962; Rogers and Balish, 1977). Influx of the highly candidacidal PMN into the liver (Louria et al., 1960) immediately after trapping and the subsequent killing of the yeasts accounts for the loss in viability seen in normal rat liver after 60 min (Table 1). This would explain observations by others indicating that liver populations of *C. albicans* decline rapidly after injection. Failure to recognize the activity of PMNs in the liver may also explain many assumptions that hepatic candidacidal activity is due to Kupffer cells (Meister et al., 1977a, 1977b). Blood monocytes may also emigrate into the liver, as shown by North (1970) using *Listeria monocytogenes*, develop into macrophage and contribute to the elimination of yeasts.

It is unknown if lymphocytes play a role in this response in normal hepatic tissue.

Clearly, the normal rat liver does not interact with *C. albicans* in a manner analogous to its interaction with bacteria. The second objective of this study is to ask whether hepatic tissue can be manipulated so that it would kill trapped yeast cells. Ideally the manipulatory process would be nonspecific enough to insure an optimal response by hepatic tissue. Characterization of this enhanced nonspecific response would of necessity have to differentiate between activity due to PMN only, as opposed to activity due to Kupffer cells. The immunopotentiator *Corynebacterium parvum* was chosen as it is known to be an activator of the MPS. It was theorized that stimulation of rats *in vivo* would be manifested by enhanced *in vitro* Kupffer cell activity.

In vivo bloodstream clearance data showed that when *C. albicans* was injected into *C. parvum*-treated rats yeast cells were rapidly cleared fromthe peripheral blood by 30 min (Table 7). Organ distribution data showed that the percentage of viable cells distributed among lungs, spleen, kidneys, and blood were not statistically distinguishable from the distribution in normal rats the total *in vivo* loss in viability is reflected primarily by the loss of viable yeast cells in the liver alone.

To obviate the possibility that killing *in vitro*

by *C. parvum*-treated hepatic tissue could be due to peripheral blood PMN only plasma was used to supplement perfusion medium in all of the remaining experiments. Moon et al. (1975) found that extended perfusion times in the absence of humoral factors increased the ability of the perfused rat liver to kill *S. typhimurium*. Killing *in vitro* was the same as that observed *in vivo* when perfusion medium contained whole blood or plasma but not blood cells. Both antibody opsonins and complement derived serum factors participate in Kupffer cell killing (Ruggiero et al., 1977). Leijh et al. (1977), in a comparison of the kinetics of phagocytosis and killing of *C. albicans* by PMS and blood monocytes (BM), found that both cells phagocytize yeasts at equal rates for the first 30 min. Yeasts are killed during the next 30 min after ingestion. Extended perfusion times in *C. parvum*-treated rat livers might increase the models' sensitivity in detecting *C. albicans* killing.

Perfusion of *C. albicans* into livers from *C. parvum*-treated rats was candidacidal both in the absence and presence of plasma. Killing was enhanced by humoral factors. Plasma did not alter the ability of the liver to trap yeasts but enhanced killing. The optimal response was detected two days after injection of 350 µg *C. parvum*. The 44% loss of viable yeasts *in vivo* (Table 5) was approximated by a 34% loss in viable yeast cells

in vitro (Table 6).

Intravenous injection of *C. parvum* results in high numbers of bacteria in the liver (Demitrov et al., 1976). Inflammation following hepatic clearance of *C. parvum* could result in the accumulation of large numbers of PMNs and BMs in the liver. These cells would in all probability adhere to liver sinusoids and not be washed out of the liver prior to infusion of *C. albicans*. They are washed out of normal rat liver prior to infusion of yeasts. Killing could be due to the PMN and/or BM derived macrophages. The maximum response of *C. parvum*-treated hepatic tissue occurs at two days. At this time the meximum PMN and BM response to *C. parvum* in the liver would be expected to occur. Perfusion studies indicated that *in vitro* killing only approximates *in vivo* killing further strengthening the possibility that phagocytic cells other than Kupffer cells might be in the liver.

In addition to stimulating macrophage directly *C. parvum* also stimulates T and B lymphocytes directly (Bomford and Christie, 1975; Christie and Bomford, 1975; Tuttle and North, 1975a, 1975b). Both events could generate activated lymphocytes and macrophages which release nonspecific lymphotoxins (Cerottini and Brunner, 1977; Pearsall et al.,1974) or macrophage cytotoxins (Ghaffar and Cullen, 1976; Lohman-Matthes, 1973, 1976; Melson et al., 1974). The mechanism of *C. albicans*

killing by *C. parvum*-treated hepatic tissue might not be due to phagocytic killing. To further investigate this possibility perfusion studies were performed in *C. parvum*-treated rats given metabolic inhibitors of phagocytic activity.

Plasma chelated with 10.5 mg EDTA lost the ability to enhance hepatic killing. Plasma ions are essential for both phagocytic engulfment of microbes (Stossel, 1974a) and for complement activation. Both activities are inhibited by chelation of essential plasma divalent ions, such as Mg^{++} and Ca^{++}, from the perfusion medium. Our data suggests that ions are necessary for the enhanced effect of plasma on hepatic killing. Whether complement alone is involved was not clarified by the use of EDTA. Further, use of EDTA does not indicate what type of cell kills *C. albicans*, the PMN or macrophage.

To clarify this point, perfusion studies with 1 mM PB and 1 mM IAA were performed to determine whether killing of yeasts was due to phogocytosis. Both PB and IAA completely abrogated the killing of trapped yeasts in *C. parvum*-treated rat liver. Leijh et al. (1977) showed similar results *in vitro* with *C. albicans*. Phenylbutazone specifically inhibited intracellular killing of yeasts and IAA specifically blocks phagocytosis of *C. albicans*. When coupled with the EDTA data (Table 9) the results indicate that killing of trapped yeasts was

not due to extracellular white cell toxins.

Friedman and Moon (1977) showed that crystalline silica is a Kupffer cell or macrophage-specific cytotoxin in the perfused liber. Silica treatment of rats *in vivo* kills cells of the MPS. The PMN population remains functional. Injection of silica abolished the ability of hepatic tissue from *C. parvum*-treated rats to kill *C. albicans*.

Cumulatively the studies with inhibitors suggest that *C. parvum* stimulated liver macrophage, and not PMN, phagocytized and killed hepatically trapped *C. albicans*. A consistent feature of *C. parvum* treatment was a relative monocytosis. Blood monocytes were elevated four fold over normal values in *C. parvum*-treated rats. Injection of silica into *C. parvum*-treated rats depressed this monocytosis. Silica treatment either depresses the release or kills the monocytes when they are released from the bone marrow. North (1970) and Volkman (1976) observed that when *L. monocytogenes* is cleared by the liver a rapid influx of peripheral blood monocytes into the liver occurs. These cells are indistinguishable from Kupffer cells when they take up residence in liver sinusoids as examined by light microscopy. Killing of *C. albicans* in *C. parvum*-treated rats could be due to newly recruited blood monocytes. They would have enhanced nonspecific phagocytic killing activity. To further clarify this possibility,

extensive SEM investigation of hepatic tissue from *C. parvum*-treated rats and from *C. parvum*-treated rats perfused with *C. albicans* was performed.

SEM of liver from rats treated with *C. parvum* revealed striking morphological differences when compared to the anatomical features of normal rat liver (Figure 2). Forty eight hours after injection of the bacteria there was a massive influx of white blood cells into the liver. This influx coincided with a relative monocytosis (Table 8). White cells adhere to the walls of portal veins including large and small branches of the portal vein, portal venules, and sinusoids. White cells adhering to portal veins appeared to be macrophages and lymphocytes (Figure 6 and 7). The morphological features of T and B lymphocytes, blood monocytes, and PMN have been studied in detail by SEM (Barber and Burkholder, 1974; Noonan and Riddle, 1977; Polliack et al., 1973; Wetzel et al., 1973). Nielsen et al. (1974) and Werdelin et al. (1974) employed SEM to study macrophage-lymphocyte cluster formation during the *in vitro* immune response to soluble protein antigen. Roelants (1977) discusses this model and the significance of macrophage-lymphocyte clustering in detail. All of these SEM studies indicate that it is not all clear, based on SEM alone, that white cells can be distinguished. It is agreed that cells with characteristic surface morphologies may be generally categorized as

either macrophages or lymphocytes. For purposes of discussion cells morphologically similar to those cells described by these studies will be referred to simply as macrophages and lymphocytes.

The cells with a satellite appearance and numerous surface folds adhering to portal vein walls appearing to be macrophage are probably of blood monocyte origin. Observations were made that the macrophages characteristically adhere to the wall with the largest portion of the cell body or "head", upstream or facing the flow of blood. A long "tail" extends downstream from the head of the macrophage. This head-tail orientation was seen most often in larger portal veins and not in smaller veins. The reasons for such an orientation are not readily apparent from the literature. The greater volume of fluid in the larger veins may influence such an orientation. Such orientation of macrophages might also be due to motility (Barber and Burkholder, 1974).

Macrophages in the sinusoids of *C. parvum*-treated rat livers have morphological features similar to those described for macrophages adhering to portal veins and to normal rat liver Kupffer cells. They occupy a significant portion of the sinusoidal lumen, have folded surfaces, stellate appearance and fine cytoplasmic processes anchoring them to the endothelium. Are they the original, resident population of Kupffer cells or are they blood

monocyte derived macrophage? North (1970) could only make this distinction by the use of autoradiographic studies of characteristic labeling patterns. Volkman 1976) showed that Kupffer cells, contrary to previous data (van Furth et al., 1975), are not derived from blood monocytes. They are a resident, self-sustaining population of cells. For purposes of discussion cells morphologically identifiable as macrophage bound either in the sinusoid or in the portal veins will be referred to simply as macrophage.

There is a considerable amount of contact or interaction between adhering white cells in *C. parvum*-treated liver. For example, the macrophages in Figure 7 have trapped lymphocytes. Macrophage-lymphocyte contact was envisaged to occur in two ways represented. Lymphocytes appear to sit in a cup-like depression on the surface of the macrophage with numerous fine cytoplasmic processes surrounding them. In the second type of interaction cytoplasmic processes extend between the surface of the macrophage contacting the surface of the lymphocyte. It is unknown if these relationships have any functional significance or if they contribute to the enhanced killing ability of *C. parvum*-treated hepatic tissue.

When compared to the ultrastructural morphology of normal rat hepatic tissue (Figure 2) it becomes apparent that when *C. albicans* enters hepatic tissue from

C. parvum-treated rats (Figure 6, 7, and 8) it encounters a liver strikingly dissimilar, functionally and morphologically, from normal liver. Microscopic examination of the inoculum prior to perfusion, and of the effluent after perfusion revealed only yeasts, blastospores and rarely a germ tube. Yeast cells observed in the perfusion medium were not aggregated, either before or after perfusion, and there was no change in the number of CFU recovered from the effluent if the effluent was homogenized or held on ice prior to plating.

Yeast cells formed large aggregates or "yeast clusters" only in perfused *C. parvum*-treated rat livers. Yeast clusters adhere to the walls of portal veins and venules. Portal vein walls contain large numbers of yeast clusters. Yeast clusters consist of numerous tightly adhering yeast cells, blastospores, and an occasional germ tube. They were entangled by many fine cytoplasmic filaments. SEM showed yeast clusters with cytoplasmic filaments and associated macrophages, yeast clusters alone adhering to portal vein walls, and yeast clusters with or without cytoplasmic filaments but with numerous macrophages. The macrophages either adhered to the clusters surrounding them or phagocytized yeasts.

Observations made by comparing SEM of clearance in normal and *C. parvum*-treated rat liver indicated that the trapping mechanism in *C. parvum*-treated hepatic tissue

was different from normal liver trapping. In normal rat liver, in the presence of plasma, trapping occurred primarily in sinusoids and not in portal veins. Yeasts back up into portal veins but they occluded the lumen of the vessel and did not adhere to the walls in any great numbers. Yeast clustering in which cytoplasmic filamentation occurred was in the *C. parvum*-treated rat. Friedman (1978, personal communication) has observed similar cytoplasmic filaments entangling *S. typhimurium* in *C. parvum*-treated mice. In the *C. parvum*-treated rat liver a significant portion of trapping appears to occur by yeast cluster formation on the walls of the portal vein.

The origin of the cytoplasmic filaments attached to *C. albicans* is unknown. They were not present in the inoculum. *Candida albicans* forms filaments *in vitro* which are 0.8-1.3 microns in diameter, but, only after 18 h of culture (Yamaguchi et al., 1974). There are no reports of *in vivo* filamentation. When coupled with the observation that these filaments are seen with *S. typhimurium* trapping and *C. albicans* trapping it may be postulated that they are of host origin. This conclusion does not conflict with reports of macrophage filamentation *in vitro* (Barber and Burkholder, 1974).

Log jam trapping of yeasts in sinusoids and portal venules, similar to log jamming in normal livers, also

occurs in *C. parvum*-treated liver. Unlike yeast cells trapped in normal liver sinusoids, yeasts in *C. parvum*-treated liver sinusoids usually were attached to cytoplasmic filaments or were being phagocytized. It may be that as yeast cells contact macrophages adhering to the portal vein walls they temporarily stick to the macrophage surface. If they were pulled away, by the flow of perfusion medium or by the impact of other yeast cells, they might draw out a thin cytoplasmic filament from the macrophage which would break off. The yeast-filament would be carried into the liver eventually ending up in a yeast cluster or in a sinusoid. Whatever their origin, filaments do not increase trapping above that seen in normal liver. Another explanation for their origin might be that they are formed by some interaction of *C. albicans* with a factor unique to *C. parvum*-treated rat plasma.

Yeast clustering could artifactually alter viability counts when plasma is added to the perfusion medium. The loss in viability in *C. parvum*-treated rat liver would then be an artifact of aggregated yeast clusters. In all of the experiments using inhibitors of phagocytosis 100% of the inoculum was consistently recovered suggesting that the loss in viability was not an artifact.

SEM studies did not resolve the question of which cell, the resident Kupffer cell or the blood monocyte derived macrophage, was responsible for killing

C. albicans. Both cells were obviously present and indistinguishable in *C. parvum*-treated livers while only Kupffer cells were present in normal rat liver. Since normal hepatic tissue does not kill *C. albicans* while *C. parvum*-treated macrophages kill the yeast one conclusion is that Kupffer cells do not participate in the hepatic clearance of *C. albicans*.

Several studies (Bomford and Christie, 1975; Christie and Bomford, 1975; Scott, 1972; Tuttle and North, 1975a, 1975b) have investigated the *in vivo* and *in vitro* mechanism of action of *C. parvum*. No studies to date have evaluated the initial response of the host to *C. parvum* treatment. Data obtained in this study indicate that early in the hosts' exposure to *C. parvum* the MPS is nonspecifically stimulated, in the absence of lymphocyte-mediated stimulation. This stimulation results in increased hepatic killing ability which may play a significant role in antimicrobial resistance.

This study characterized the initial hepatic clearance of *C. albicans* by normal rat hepatic tissue. The mechanism of clearance was different from that of bacterial hepatic clearance. Clearance of yeasts occurs primarily by nonphagocytic trapping in liver sinusoids. *In vivo* and *in vitro* pathophysiological experiments suggest that sinusoidal trapping was the major way in which yeasts were cleared. SEM confirmed this. Treatment of rats with

C. parvum resulted in an influx of white blood cells into the liver. Pathophysiological experiments showed that the macrophages present in these adhering white cells killed the yeasts. Killing occurred optimally in the presence of humoral factors. Characterization of this altered hepatic function suggested that *C. parvum* enhances nonspecific antimicrobial resistance by increasing the ability of hepatic tissue to destroy trapped microbes. If the hypothesis that the increased killing ability resides in newly recruited blood monocyte derived macrophages is correct, it may be concluded that in both normal and *C. parvum*-treated rats, Kupffer cells play a minor role, if any, in the hepatic clearance of *C. albicans*.

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