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METABOLIC PATHOGENESIS OF *CANDIDA ALBICANS* IN MICE

By

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ABSTRACT

METABOLIC PATHOGENESIS OF CANDIDA ALBICANS IN MICE

By

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Pathophysiologic responses in mice were studied after intravenous injection of either 1.0×10^6 or 4.5×10^6 cfu C. albicans. Mice challenged with 1.0×10^6 C. albicans died between 1 and 16 days after infection. Tissue distribution studies revealed that in these mice near the time of death, over 98% (2.7×10^7) of the fungi recoverable from six major host organs was localized in the kidneys. This large renal tissue burden was responsible for severe impairment of renal function as evidenced by elevated BUN and blood creatinine values in infected mice. The magnitude of the elevations over normal values was comparable to that seen for bilaterally nephrectomized mice near death. The creatinine clearance rate of infected mice was about one-half that of normal mice. No abnormalities in carbohydrate metabolism were detected in infected mice by assaying liver glycogen and blood glucose at 48 hours after challenge and near the time of death.

All mice receiving 4.5×10^6 cfu C. albicans died by 12 hours after challenge. These mice showed hyperglycemia and normal BUN values near the time of death. Liver glycogen, blood glucose, and BUN for mice receiving 4.5×10^6 heat-killed C. albicans cells (ip) were normal 24 hours after challenge, and for mice receiving 150 ug of S. typhimurium

endotoxin, liver glycogen and blood glucose were lower and BUN was higher than normal.

Two distinct syndromes were observed for the two challenge doses. At the lower dose, mice seemed to be dying from renal failure associated with a severe renal fungal infection. Response to the higher dose was not as well understood but if a toxin was associated with death from this dose, it was not similar to bacterial endotoxin.

To Thea,

for your patient understanding

and constant encouragement.

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INTRODUCTION

In recent years, there has been a significant increase in the incidence of human infection by the opportunistic pathogen Candida albicans (12, 21, 22, 59, 69, 85). Debilitated hosts such as post-surgical patients (10, 59, 61, 68, 83, 84), leukemia and other cancer patients (45, 59, 61, 70, 75, 83, 84, 87), patients with diabetes mellitus (45, 49, 83, 84), patients with indwelling catheters and/or on parenteral hyperalimentation (10, 49, 59, 61, 87), and patients on longerterm antibiotic (22, 49, 59, 61, 84) or corticosteroid (22, 49, 59, 70, 75, 83, 84) therapy all run the risk of developing systemic candidiasis. Most disseminated infections by C. albicans are nosocomial (22, 61).

Animal models have yielded considerable information regarding systemic infection by C. albicans. Animals used include rabbits, guinea pigs, rats, and mice. The mouse model has been used extensively to determine the relative pathogenicity of several isolates of C. albicans as well as other members of the genus Candida (23, 37, 50, 51, 53, 72). The predilection of C. albicans for progressive infection of the kidneys has been established (14, 48, 50, 64). Histopathology in several organs has been observed and inflammatory responses have been studied (1, 3, 48, 50, 79).

Much remains to be learned about systemic infection by C. albicans. The virulence factors of the organism have not been clearly

identified. Whether the yeast or mycelial phase of this dimorphic fungus is the invasive form is unclear. The rapid development of mycelium *in vivo* (30, 79, 86), the abundance of mycelium observed in tissues (19), and the behavior of mycelium in leukocytes (50) would suggest an invasive role for the hyphal phase of the organism. By contrast, the lack of virulence of stable mycelial variants and yeasts transformed *in vitro* (14, 52, 56, 71) would suggest that the yeast phase also has a significant role in establishing an infection.

The existence of a toxin has been postulated, based primarily on the rapid mortality of infection with a large number of organisms (26, 34, 58, 67, 80, 82) and on toxic activities of culture supernatants (50, 53, 54, 55) or fractions of cells (11, 39, 40, 41, 58). Unfortunately, no specific toxin has yet been purified from a large number of strains.

Debate continues as to exactly where *C. albicans* has its lethal effects in the host. The kidney is the organ most heavily infected, but other organs show frequent and severe involvement (6, 22, 49, 59, 61, 69, 83). Significant impairment of renal function has been observed (80, 81) but, to this point, renal failure has only been assumed. Some recent evidence points to serious cardiac damage during infection (60).

Surprisingly little is known about the metabolic and physiologic alterations accompanying systemic candidiasis. In this study, the parameters of liver glycogen, blood glucose, blood urea nitrogen (BUN), and creatinine clearance are examined. Any abnormalities might suggest that untoward effects on carbohydrate metabolism may be significant in pathogenesis. A similar approach has been used in studying the pathogenesis of endotoxin (5). BUN and creatinine clearance are standard measures of renal function and are used to assess renal damage during

infection (15, 29, 46). Cumulatively, the data suggest that renal damage is much more severe than altered carbohydrate metabolism in murine candidiasis.

LITERATURE SURVEY

Candida albicans and Human Candidiasis

C. albicans belongs to the Form-Class Deuteromycetes because its primary mode of reproduction is asexual budding. It is further classified in the Form-Order Moniliales (conidia not borne on specialized structures or reproduction by oidia or budding) and Form-Family Cryptococcaceae. C. albicans exists in a variety of morphological forms (83). It is commonly observed as a yeast or blastospore which reproduces by budding. The yeast can elongate to form mycelium, also called pseudomycelium or pseudohyphae, which can produce blastospores or more mycelium by budding. Chlamydospores, thick-walled and larger than blastospores, are also produced by the mycelial phase and are found terminally on the hyphal strands.

C. albicans is part of the normal flora at many sites on the human body. It is a saprophyte on mucocutaneous surfaces and is isolated without apparent relation to disease. It has been isolated from the mouth and oral cavity of healthy adults at frequencies ranging from 6-30% (83, 84). One study reported C. albicans isolation from normal sputum samples at a rate of 36% (4). C. albicans also resides in the gastrointestinal tract and is therefore commonly present in feces (83, 84). Carrier rates for the gastrointestinal tract have been reported at 15-20% (32). One study reports that C. albicans maintains a stable gut population which increases in frequency and concentration from the

oropharynx to the colon (9). C. albicans also inhabits the vagina of the normal, non-pregnant female at rates ranging from 5-10%, and this frequency substantially increases during pregnancy (83, 84). In one report, C. albicans was isolated, usually at low density, in urine specimens at a frequency of about 10%. It was isolated two times more frequently from females than from males and was observed to be the most common yeast found in human urine specimens (2). C. albicans is very infrequently isolated from the skin and is probably only a transient inhabitant of normal skin (83). Animals of many kinds are carriers of C. albicans, primarily in their digestive tracts, and the few isolations of C. albicans from the soil most likely represent fecal contamination (83).

Since C. albicans commonly colonizes the human body as a saprophyte, any condition which disrupts the balance between host and parasite leads to a pathological state. C. albicans is thus an opportunistic pathogen, causing disease only under adverse or abnormal conditions. It can take advantage of any opportunity which would favor its development and propagation (70).

Candidiasis refers to any infection by a member of the genus Candida. The disease includes a wide variety of clinical presentations and can manifest either as an infectious or an allergic phenomenon. Candidiasis can be localized or systemic, superficial or deep, acute or chronic, and involve almost any tissue. It has no preference for any age, race, or sex, and occurs worldwide (45, 61). The recognized clinical patterns of infectious candidiasis can be grouped into three categories: those showing mucocutaneous, cutaneous, or systemic involvement. Mucocutaneous forms of candidiasis include thrush,

perléche, vaginitis, balanitis, bronchial and pulmonary candidiasis, alimentary candidiasis (esophageal, enteric, and perianal), and chronic mucocutaneous candidiasis. Cutaneous forms may involve the axillae, the intermammary folds, the umbilicus, the interglutal folds, inguinal areas, interdigital spaces, and paronychial folds. Systemic candidiasis may involve any or all of the major organs or may be limited to the urinary tract, the heart (endocarditis), the central nervous system (meningitis), or the bloodstream (septicemia). Allergic phenomena include candidids, eczema, asthma, and gastritis.

Systemic candidiasis is one form of candidiasis which has increased substantially in recent years (21, 69, 85). Although candidiasis is not a specific notifiable disease, the number of deaths per year in the U.S. from acute candidiasis has been monitored (57). For the period 1950-1959, the average number of deaths per year was 86.0; for 1960-1969, 98.2; and for 1970-1975, 171.3. In twenty-one of these twenty-six years, candidiasis was the leading cause of mycotic deaths in the U.S. One survey of hospital records shows a five-fold increase in the number of patients with deep Candida infections from the period 1960-1963 (7 cases) to 1964-1967 (35 cases) (22). Another survey of hospital autopsy records reports a six-fold increase in occurence of systemic candidiasis for the period 1971-1975 (6.8 cases per year) compared to the period 1963-1970 (1.1 cases per year) (59). Symmers notes that this increase is not just a reflection of better diagnosis (75). Selliger agrees and attributes the increase to technical and therapeutic advances which prolong and save lives (70). Use of antibiotics, corticosteroids, and life-sustaining equipment predisposes patients to

systemic Candida infection and, in effect, provides more debilitated hosts in which infection can occur (69).

As an opportunistic pathogen, C. albicans causes disease in the compromised or debilitated host. Numerous factors have been identified as predisposing one to Candida infection. Extreme youth, with absence of a resident flora, can lead to thrush in the infant (83, 84). Maceration of the skin (84) or trauma, such as burns (61), can allow colonization of the skin and subsequent cutaneous infection. Pregnancy (83, 84) and use of oral contraceptives (70) can predispose to vaginitis as reflected in both increased glycogen content of the vaginal epithelium and increased frequency and numbers of yeasts (70). Obesity and malnutrition (83, 84) can also predispose to Candida infection.

Systemic candidiasis has several additional predisposing factors. Injection or infusion of contaminated solutions, use of contaminated eye and ear drops, or contaminated equipment such as respirators and hemodialysis equipment can give rise to a Candida infection (70, 75). Heroin usage has been linked to Candida endocarditis (45). Catherization (61, 70, 87) and other long-term intraveneous therapy (49, 59, 61, 87) show a high correlation with development of Candida septacemia and deep tissue infection.

Long-term antibiotic therapy, particularly with broad spectrum antibiotics, is a factor of prime importance in the development of systemic disease (22, 49, 59, 61, 77, 84). The antibiotic suppresses the competing bacterial flora and allows an overgrowth of Candida to occur (6, 69, 70, 83). The drug may actually damage the mucous membrane and allow easier penetration of the organisms (69, 70). There have been

some reports that certain drugs have a direct stimulatory effect on Candida but this is still a controversial matter (6, 49, 83).

Similarly, administration of corticosteroids and other immunosuppressive drugs is an important factor in predisposing to systemic candidiasis (22, 49, 59, 70, 75, 77, 83, 84). Cortisone has been shown to retard clearance of Candida from all tissues in vivo and allow significant proliferation of the fungus in the kidneys during this period (48, 49).

Surgery, especially abdominal and heart surgery, with postoperative antibiotic prophylaxis is a notable predisposing factor (59, 61, 70, 75, 77, 83, 84). Abdominal surgery can provide a portal of entry for hematogenous spread of the fungus (6).

Any of a number of debilitating diseases (49, 75, 87) or bacterial infections (59, 61, 75, 77) can compromise the host so as to allow a Candida infection to occur. Notable among these is diabetes mellitus (45, 49, 83, 84) in which increased blood glucose is an energy source for growth of the organisms (70). Cancers and neoplasias (45, 61, 70, 77, 83, 84), especially hematologic malignancies such as leukemia (59, 70, 75, 77, 83, 84, 87) are important predisposing factors for systemic candidiasis. Cytotoxic drugs (22, 59, 61, 70, 75, 87) or radiation therapy (22, 83, 84), used to treat these diseases, are also involved.

Most often, it is a combination of several of the above factors which allows a Candida infection to occur. For example, debilitation can predispose but rarely was systemic candidiasis observed in patients with underlying diseases or malnutrition before the use of antibiotics became widespread (49, 69). Because systemic candidasis is often a

secondary infection, complicating a previously existing disease, it is understandable that many cases are nosocomial (22, 61).

The portal of entry for the fungus is related in some degree to the predisposing factors. Invasion of Candida through the gastrointestinal tract, where it is a commensal, has been shown histologically to occur (61) and has often been cited as the most frequent portal of entry (61, 68, 69, 83). Braude and Rock noted a high incidence of gastrointestinal bleeding or injury in patients with disseminated candidiasis (6). Contaminated intravenous lines or introduction of contaminated substances is also a common portal of entry (61, 75, 83, 87).

Several surveys of case reports of human candidiasis have appeared in the literature (6, 22, 49, 59, 61, 69, 75, 83, 87). These describe the general features of the disease. The most frequently reported clinical signs include fever (6, 37, 49, 75, 77, 83) and impaired renal function as evidenced by increased BUN (6, 22, 59, 77). Oral thrush (75, 83), chills (49), hypotension (6, 49), splenomegaly (49, 75), leukopenia or granulocytopenia (22, 59, 77, 87), leukocytosis (75, 77), and depression of sensorium or confusion (6, 83) have also been reported depending on the sites and severity of infection. Myerowitz et al. note that clear clinical or microbiological evidence of infection may appear late during the course of disease or not at all (59).

Diagnosis of systemic candidiasis is not easy. Traditionally, diagnosis has been made by culture of the organism; however, candidiasis of deep tissues does not consistently yield positive blood cultures. In one study of patients with Candida septacemia, Candida was recovered more than once from less than half (19/43) of the subjects (77). In another report, positive blood cultures were obtained in only 16/39

patients with disseminated candidiasis (59), and in still another, only 21/35 patients had positive blood cultures (22). Parker et al. reported that in twenty-five patients with deep candidiasis, no positive cultures from any site were obtained from four patients (61). Young et al. warn that, when detected, some fungemias are only transient so every case should not necessarily be treated. They note that candidemia in the compromised host is most likely indicative of deep infection if associated with immunosuppressive therapy, leukopenia, and absence of an intravenous catheter (which tends to promote transient fungemia, curable by removal of the catheter) (87).

In a study of twenty-nine patients, diagnosis of deep candidasis was made in 14 of 29 by two or more positive blood cultures, in 4 of 29 by positive cultures reported after death, in 5 of 29 by positive cultures taken at autopsy, and in 6 of 29 patients no positive cultures were obtained but the presence of fungi in tissues was observed at autopsy (49). Unfortunately, many diagnoses of systemic candiasis are made at autopsy (42, 75, 87). Some authors stress the importance of culturing additional sites besides the blood such as urine, sputum, bronchial washings, peritoneal fluid, or pleural fluid (22, 49, 87).

Serum antibody titers and delayed type hypersensitivity (DTH) reactions have been of little or no diagnostic significance in the past. Because of the high carrier rate among normals, high antibody titers and DTH reactions to Candida antigens are common in the general population (18, 32). Recently, however, some progress has been made toward the development of reliable immunologic methods of diagnosis. Taschdjian et al. (76), found a test for precipitins to a cytoplasmic "S" antigen to be 88% effective in diagnosis of deep Candida infection

but the method gave no indication of the extent or severity of disease. Immunodiffusion and latex agglutination have been applied to the diagnosis of systemic candidiasis with some success, but these methods are not without problems (42).

Autopsy study of patients with disseminated candidiasis has yielded information about the pathology of the infection. The kidney is the organ most severely and most frequently involved (49, 59, 61, 69, 83). The brain (6, 61, 69, 83), lung (22, 49, 61), liver (59, 69), heart (6, 49, 61), and GI tract (22, 61) are also frequently involved. Other organs including blood vessels (61, 69, 83), spleen (22, 49, 59, 61, 69, 83), thyroid (6, 22, 49, 61, 69, 83), prostate (22), peritoneum (61), pancreas (49, 61), adrenals (6, 49, 61), ureters (61), epiglottis (49), larynx (49), and bladder (49) have been involved to a lesser degree.

The tissue lesions of systemic candidiasis have been described histopathologically by many authors (6, 49, 59, 61, 83). With severe, rapidly fatal infection, most organs show many microabscesses (83) containing both yeast and mycelial forms (6, 58, 83). An inflammatory response ranging from an acute suppurative reaction with the presence of many neutrophils to mild infiltrates of PMN's and mononuclear cells (61) or small masses of fungi surrounded by histiocytes (83) may be present although several instances have been reported in which there was no cellular infiltrate (49, 83). Lack of an inflammatory response did not correlate with the presence of diseases like leukemia, or granulocytopenia; in some cases, a significant PMN response was seen in different section of the same tissue (19). Intra- and extracellular yeasts and mycelia have been reported, as has a lack of phagocytic

activity (6). Occasionally, granulomatous lesions with giant cells and histiocytes have been observed and denote a more chronic infection (61).

Amphotericin B is the most effective drug for the treatment of disseminated candidiasis (49, 77, 83). It is administered intravenously at a usual dose of 1 mg/kg body weight/day for long periods of time. It has several serious side effects including chills, fever, nausea, vomiting, and anorexia. It is nephrotoxic and treatment must be discontinued if kidney function is severely impaired.

Another drug with antifungal activity is 5-fluorocytosine (31). It is more convenient than amphotericin B (it is taken orally) and is less toxic, but it is also less effective in treatment.

Clotrimazole appears to be quite active against most pathogenic fungi but was not very successful in trials with mice because of adaptive hepatic catabolism of the drug. Also, absorption is erratic following peroral administration and severe nausea and vomiting often result (31).

Even with treatment, systemic candidiasis is generally fatal, whether due to the severity of Candida infection or the severity of underlying disease (49, 69).

Animal Models of Candida albicans Infection

Animal models have been used extensively in the study of systemic candidiasis. Modern research on the pathogenicity of the genus Candida for laboratory animals began with attempts to reproducibly establish fatal systemic infections.

Fuentes et al. (16) gave C. albicans intravenously (10^6 cells/100 g of weight) to rats, mice, guinea pigs, and rabbits. The dose was fatal to 100% of the rats and mice, 95% of the rabbits, and 83% of the

guinea pigs. The average survival time was 5 days for rats and rabbits, 11 days for guinea pigs, and 15 days for mice. Sleepiness, nasal and ocular hemorrage, and paresis were common symptoms noted and the kidney, brain, and gall bladder were observed to have the most organisms although positive cultures were obtained from all organs.

Hasenclever (23) studied comparative pathogenicity of C. albicans for mice and rabbits. He concluded that, on a weight for weight basis, the susceptibility of the mouse to candidiasis was equal to or greater than that of the rabbit. The LD_{50} of isolates of C. albicans for the mouse ranged from 1.5×10^2 to 1.4×10^4 cells per gram of animal weight and for the rabbit from 4.8×10^2 to 1.2×10^5 cells per gram of animal weight.

With this early work, it was clearly established that C. albicans was pathogenic for several different laboratory animals.

Early studies of systemic candidiasis were also concerned with the varying degrees of pathogenicity of the members of the genus Candida for laboratory animals. The genus Candida contains over 25 species (17) but only seven are commonly isolated from humans. These include C. albicans, C. guilliermondii, C. krusei, C. parapsilosis, C. pseudotropicalis, C. stellatoidea, and C. tropicalis (37).

Of all the members of the genus Candida, C. albicans possesses the greatest pathogenicity for man and laboratory animals. It is the species most frequently isolated from human Candida infections (22, 37, 49, 61, 77, 87). Some studies have directly examined the varying degrees of pathogenicity of the species of Candida. Mankowski (53), using six isolates of each species, found C. albicans highly pathogenic, C. stellatoidea and C. tropicalis pathogenic to a less degree, and C.

krusei, C. parapsilosis, C. pseudotropicalis, and C. guilliermondii nonlethal when 4 X 10^6 cells were given intravenously to mice. When given intracerebrally, C. albicans, C. tropicalis, and C. stellatoidea were pathogenic, but death took longer than if given intravenously. C. krusei and C. pseudotropicalis were fatal to some mice when suspended in 5% mucin for inoculation but not when administration of cortisone or estradiol accompanied infection. C. guilliermondii was not lethal for mice in any case.

Hasenclever (23) found that, of all seven species, only C. albicans had lethal effects for rabbits at intravenous doses up to 5 X 10^8 cells. For female Swiss mice, C. albicans showed a high degree of lethality, and all other species were not lethal at intravenous doses up to 5 X 10^6 cells.

Winner and Hurley (37) found C. albicans, C. tropicalis, C. stellatoidea, and C. pseudotropicalis to be more pathogenic and C. krusei, C. parapsilosis, and C. guilliermondii to be less pathogenic for mice based on mortality after intravenous injection of 2.5 X 10^6 yeast cells.

Hasenclever and Mitchell determined that strains of C. tropicalis were less virulent than strains of C. albicans for mice and showed little pathogenicity at all for rabbits (24). Likewise, Louria et al. (50, 51) concluded that C. albicans was more pathogenic than C. tropicalis. Five strains of C. albicans produced an average of 96% mortality when injected intravenously at a dose of 5 X 10^6 cells and five strains of C. tropicalis produced an average of 70.2% mortality.

Stanley and Hurley (38, 72) observed a correlation between the differential frequency at which the species of Candida are isolated

from clinical disease in man, their pathogenicity for laboratory animals, and their ability to kill mouse renal epithelial cells in tissue culture. They ranked the species of the genus Candida in the following probable order of virulence: C. albicans, C. tropicalis, C. stellatoidea, C. pseudotropicalis, C. parapsilosis, and C. guilliermondii. The position of C. krusei is one of low pathogenicity but its exact place is ambiguous.

Thus C. albicans has emerged as the most important and most dangerous pathogen within the genus Candida.

The mouse model of experimental disseminated candidiasis has been most used and is the best understood. Mourad and Friedman (58) injected mice with C. albicans at various doses by various routes. Peroral, intramuscular, subcutaneous, and intranasal innoculation either were not fatal or caused only very few deaths at the highest of doses (1×10^8 cells). Intracerebral innoculation caused death at irregular intervals and some deaths were thought to be due to trauma. Intraperitoneal innoculation caused generalized, fatal infections at higher doses (10^6, 10^8 cells). Intravenous innoculation at lower doses (10^4, 10^6 cells) was found most suitable for causing prolonged infection. In addition, with this route, there was a dose-response relationship for both percent mortality and time of mortality.

Young (86) studied the invasion of C. albicans by injecting 4×10^7 cells intraperitoneally into mice. The yeasts were either phagocytized or rapidly formed mycelia and tended to invade monocytes. The pancreas and then the kidney were the only organs invaded by 24 hours. C. albicans persisted in the kidneys of infected mice for up to six months.

The distribution of viable C. albicans in the tissues of infected mice after intravenous injection has been determined by Louria et al. (48). They found that tissue populations in the kidneys of infected mice increased in 14-22.5% of the mice given 10^5 to 5 X 10^6 cells. In the rest of the mice, kidney populations remained stationary for up to 2 weeks before decreasing. In all other organs, there was no progressive infection observed and the organs were cleared sooner than the kidneys.

Progressive kidney infection was also shown to occur for three more virulent strains of C. albicans to a much higher degree than noted above (50). Evans and Mardon (14) showed that relatively few cells localized in the kidneys initially after injection but the ones that did, grew to cause a fatal infection. Rogers and Balish (64) showed that tissue census in the two kidneys of a mouse was not always the same, suggesting that asymmetrical clearance occurred in the kidneys of a large percentage of infected mice. C. albicans shows a marked tendency for multiplication in the kidneys as opposed to any other organ in the infected animal.

The histopathology of lesions in the mouse model of systemic candidiasis has been studied in detail. After injection, blastospores become trapped in the capillaries of kidneys, heart, liver, lungs, spleen, brain, pancrease, adrenals, and skeletal muscle (48). Organisms, both mycelial and yeast forms, penetrated into tissues in 2 to 10 hours (48, 50, 79). An inflammatory response is evoked and abscesses are formed between 16 and 30 hours (48, 50). Microabscesses have been reported in the kidneys, brain, heart, liver, pancreas, and skeletal muscle but are rarely or never reported in the lung, spleen, and

adrenals, although there may be diffuse infiltrations of inflammatory cells in these organs (1, 48, 50, 79). In all organs except the kidneys, lesions begin to regress and few organisms can be found by 2 or 3 days (48, 50, 79). Winblad did note progressive infection in the brain and heart as well as the kidney in some cases (79). Most attention has been paid to the kidney pathology (1, 3, 48, 50, 79). In 1 to 2 hours after injection, yeasts are observed in glomerular capillary loops and cortical interstitial capillaries (50, 79). Mycelial development and growth occur and the fungus penetrates into the renal parenchyma of the cortex. With this comes interstitial infiltration of lymphocytes (1) or neutrophilic granulocytes (79) and histiocytes (1, 79) and subsequent abscess formation around masses of organisms. Fungal multiplication continues and penetrates into the kidney tubules where growth of long hyphal forms continues unimpeded and no inflammatory response is evoked (48, 50, 79). From the tubules, the fungus penetrates back into the tissue of the cortex and medulla, eliciting a marked inflammatory response consisting mostly of PMN's, and formation of large abscesses (3, 48, 50, 79). In some cases, granulomas are observed in the cortex and medulla, consisting of histiocytes surrounded by a zone of lymphocytes. No fungi are usually visible in these lesions and they generally indicate a more chronic condition (1, 79). If the animal survives infection, the abscesses regress in 1 to 2 weeks, leaving only scars (48, 50).

Hurley and Winner (36) described renal candidiasis as a variant of systemic disease involving a chronic infection with lesions localized in the kidneys. In the early stages, the kidney lesions are similar to those of acute systemic disease. In the later stages, the

cortex remains scarred and the renal pelvis becomes the seat of a large mycelial mass with hydronephrosis and renal atrophy. Similarly, Winblad (79) observed distinct acute and chronic types of disease with the chronic type leading to urinary blockage and hydronephrosis. Both investigators noted unequal involvement of the two kidneys in one animal (36, 79). Hurley and Winner (36) also recorded human cases of candidiasis strictly limited to the kidneys.

One group assayed serum constituents in an effort to identify biochemical alterations reflecting the histopathology of systemic candidiasis in mice (60). They observed increases in blood urea nitrogen, creatine phosphokinase, glutamic oxalacetic transaminase, glutamic pyruvic transaminase, and lactic dehydrogenase in the serum of infected mice. Histological examination showed serious tissue damage only in the heart, and they concluded that the histopathology and altered serum biochemistries of infected mice correlated with one another.

The mouse model of systemic candidiasis has also been used to test the effect of steroids on Candida growth and infection. Cortisone has been shown to increase total mouse mortality, enhance renal involvement, and delay clearance of Candida from extra-renal sites in systemic infection (48).

Systemic infection by species closely related to C. albicans has been studied using the mouse model. C. tropicalis, like C. albicans, infects the kidney to the greatest degree (24, 51). C. tropicalis shows a greater tendency for progressive multiplication in the brain than C. albicans, but aside from this unusual host reaction in the brain, the pathology of infection is the same for the two species (24, 51). Steroid administration with infection increases C. tropicalis

populations in lung, liver, spleen, heart, kidney, and brain but increases C. albicans tissue population only in the kidneys (51).

C. parapsilosis and C. guilliermondii are considerably less pathogenic than C. albicans (17, 23). After intravenous injection, neither organism produces a progressive infection in any organ and tissue populations steadily decrease with time (17). With steroid treatment, C. parapsilosis progressively infects the kidneys and clearance from the brain is delayed (17). The inability of C. parapsilosis and C. guilliermondii to produce disease appears to be related to their inability to form mycleium and invade renal tubules. Pathologically, neither organism in tissues elicits an inflammatory response (17, 60).

Torulopsis glabrata, a yeast which does not form mycelium, does not produce a progressive infection in normal mice but does remain viable in tissue for 8 to 10 weeks. If the host is altered by administration of cortisone, progressive infection occurs in the kidneys until steroid treatment is stopped, at which time the tissues are cleared (25).

The rabbit has also been used as a host for the production of systemic candidiasis by intravenous innoculation. The kidney is the organ most seriously involved and the pathology of infection is very similar to that observed in the mouse (13, 33). One finding not recorded for the mouse was an additional kind of renal lesion involving a hyaline thickening of the basement membrane of some capillary loops, with necrosis (13). The significance of this lesion is not known.

The guinea pig has also been used as a model for systemic candidiasis. As in the mouse model, the kidney is the most severely affected organ (34, 79, 82), and the pathology is much the same for the

two hosts (79, 82), and cortisone treatment increases mortality and renal tissue populations (35). Several notable differences exist between the mouse and guinea pig models of systemic candidiasis. Pneumonia was a significant factor early in infection (34, 82) and leukocytosis accompanied systemic infection in the guinea pig (34). Lesions in the kidney occur equally in the cortex and medulla of guinea pigs (34), but are seen more often in the cortex of mice (48). One investigator reported little discernable inflammatory response in acute systemic infection in the guinea pig (82) and another reported an impressive response (34).

The rat has been used only rarely as a model for systemic candidiasis. The kidney is the only tissue in which progressive multiplication occurs and a weak PMN infiltrate has been observed in tissues early in infection (65).

Yeast and Mycelium in the Pathogenesis of Candida albicans

That C. albicans is pathogenic for man and laboratory animals is well established. The basis of this pathogenesis, however, is not well understood. Specific virulence factors for C. albicans have not been clearly identified. Whether the yeast or mycelial form of this dimorphic fungus is the invasive or virulent form is not entirely clear. The roles of the two phases in infection have been the subject of much research and much debate.

The mycelial form of C. albicans predominates in tissue sections of lesions from infected animals and humans (19). This was interpreted by many to mean that the mycelial form is the virulent form.

Hill and Gebhardt (30) first noticed that within sixty minutes after subcutaneous injection of C. albicans yeasts into mice, almost

all of the yeasts developed short pseudohyphae. They observed that yeasts were readily phagocytized and showed signs of degradation inside phagocytes but rarely was a yeast cell with a tail ingested. They speculated that transformation favored survival of the fungus by hindering ingestion and postulated a significant role for the mycelial phase in infection.

Young's (86) observation of peritoneal smears from mice infected intraperitoneally with C. albicans yeast cells confirmed this. Yeasts were seen intracellularly and showed evidence of degeneration but mycelia appeared to invade the cells and showed no signs of deterioration. He concluded that the mycelial phase is invasive because the yeasts are readily phagocytized. Louria et al. (50) noted the ability of several strains of C. albicans to form pseudohyphae and grow out of apparently viable leukocytes after being phagocytized.

Winblad (79) studied infection in mice and guinea pigs and noted that the transformation to mycelium was connected with progressive disease only in the kidney. He supported the idea that the mycelial form is the invasive form in the kidneys.

Other experimentation has favored the yeast phase as being the invasive form. Mackinnon (52) isolated a stable mycelial variant of C. albicans which was not lethal for rabbits when injected intravenously at doses up to 5×10^8 mycelial units.

Reynolds and Braude (63) identified a factor in blood which transformed the yeast into the mycelial form. They thought that in vivo, transformation would occur to stop the multiplication of the fungus and this is why Candida infections were common superficial occurences but rarely spread systemically.

Simonetti and Strippoli (71) found that with intraperitoneal innoculation of mice with equivalent doses (by O.D.) of yeast and mycelial forms, the group given yeasts showed a higher percent mortality and shorter mean survival time than the group given mycelia. The same was true for rabbits injected intravenously. They also injected rabbits intradermally with yeasts or mycelia and reported that at low doses, the yeasts produced larger lesions than the mycelia and at higher doses, the lesion sizes were the same but those of the yeasts decreased in size slower than those of the mycelia. Their conclusion was that the yeast form was more virulent than the mycelial form.

The criticism can be made that samples of yeast and mycelium of equal O.D. do not necessarily contain the same number of viable units and this was shown to be the case by Mardon et al. (56). These investigators gave increasing doses of yeast and mycelial phase organisms to mice and found that with increasing doses of yeasts, the survival time of the mice decreased whereas with increasing doses of mycelia, the survival time remained the same. Based on this finding, they concluded that some, though not all, of the difference in mortality from yeasts and mycelia was due to the difference in morphology and thus the number of viable units of the two phases. They went on to suggest that differences in mortality from yeast and mycelial preparations may result, in part, from different localization patterns in the host.

Evans and Mardon (14) specifically addressed this proposition. After finding that 40X the amount of mycelial variant was necessary to obtain comparable lethality to that seen with the homologous yeast, they studied the distribution of yeast and mycelial forms in major host organs after injection. The mycelial variant localized to a greater

degree than the yeast in the lungs and to a lesser degree in the liver. Organisms of both forms were cleared more rapidly from the lungs than from the liver, suggesting that the lungs may deal more efficiently with the fungus than the liver. Thus the mycelial form localizes to a relatively larger degree in an organ which can more easily eliminate it. Furthermore, both forms were present in the kidneys in small numbers initially. The yeasts began to multiply within 24 hours whereas the mycelia did not show multiplication even at 96 hours. The conclusion was that the yeast form was important in initiation of infection.

When C. albicans in the yeast form is incubated in serum, there is a progressive increase in the proportion of mycelial form with time. Oblack et al. (60) found that the longer the incubation in serum, the greater the decrease in mortality after iv injection into mice. In addition, after injection of yeast or mycelia, the viable organisms in the tissues were counted. The number of viable units in all tissues 12 hours after mycelium injection was significant less than the number of viable units 12 hours after yeast injection. This suggests that a host response may contribute to the less severe infection of the mycelial phase, although biochemical differences between the 2 phases are not ruled out.

Hurley and Stanley (38) observed an association between the extent of mycelium production and the extent and severity of cytopathic effect in tissue culture of mouse renal epithelial cells. Because there was greater epithelial cell involvement by strains with rapid mycelial growth rates, they noted the importance of the mycelial phase in progression of the lesion (although there was no qualitative difference between the cytopathic effect of yeast and mycelium). They suggest

that the mycelial phase is associated with Candida species which grow more rapidly and this phase has a significant role in extension of the lesion.

The pattern that has emerged would indicate that the yeast phase is involved in initiation of infection and the mycelial phase is involved in extension of infection. Precise roles for the two forms are not clear and significant biochemical differences between the two forms, which would serve as specific virulence factors, have not been ruled out.

Toxins in the Pathogenesis of Candida albicans

It is also unclear as to whether toxins are involved in the pathogenesis of C. albicans infections. Toxin involvement has been postulated by early workers who infected laboratory animals with C. albicans (16, 53, 81). A threshold dose has been observed for rabbits (80), mice (58), and guinea pigs (34, 82), under which a long-term slowly fatal or non-fatal infection is established and over which rapid death ensues. There is no multiplication of the fungus in major organs or tissues by 12 hours after infection but most deaths from large doses occur before this time (26, 50) suggesting the production or liberation of a toxin.

Several investigators have observed that injection of culture supernatants of C. albicans has deleterious effects for the host. Louria et al. injected mice with supernatants from four week old cultures and up to 44% of the mice died within 24 hours (50). Mankowski reported that filtrates of six week cultures of C. albicans injected into C3H mice were fatal (53) and into newborn Swiss mice slow the

growth rate and cause premature aging, depilation, and glycogenosis in some of the mice (54). He isolated and partially characterized a glycoprotein which was responsible for the slowing of the growth rate. It was also toxic to Swiss mice (0.75 mg/g body weight) (55).

Other investigation has studied the toxicity of extracts of C. albicans cells. Mourad and Friedman sonically ruptured cells and found the supernate, but not the washed sediment, to be toxic for mice, causing deaths in less than 12 hours (58). Sonically ruptured cells of nonhuman isolates were also toxic, although the viable cultures were relatively harmless. On this basis, they postulated that other factors in addition to toxic substances are responsible for the pathogenesis of C. albicans.

Henrici (28) in 1940 was one of the first to speculate that a toxin akin to bacterial endotoxin might play a role in the pathogenesis of C. albicans. Endotoxic-like symptoms such as fever, shock, and leukopenia were observed in animals after the injection of C. albicans cells (58, 67). Also, toxic-like reactions such as fever, hypotension, petechial rash, and shock (6, 49) were observed in humans with acute, systemic candidiasis.

Salvin (67) injected mice intraperitoneally with killed cells of C. albicans at a high dose (10^9 cells) in adjuvant and produced a high degree of lethality. This toxic effect was observed to some degree for all six strains used, but showed no correlation to the virulence of viable cells of the strains. Salvin noted that, like endotoxin, his toxic material was not free in the medium and did not give rise to protective antibody.

Isenberg et al. concluded that a material resembling endotoxin was produced by strains of C. albicans virulent for mice after comparing ethanol-ethyl ether and phenol extracts of mouse virulent and avirulent strains (39, 40). The phenol extract from one mouse virulent strain was lethal within 24 hours to 50% of the mice tested. In survivors, there was dermal necrosis at the site of injection by 48 hours and survivors were resistent to challenge with a normally lethal dose of viable cells of the homologous strain. The phenol extract of the avirulent strain had no effects. There was evidence of cell surface differences between virulent and avirulent strains.

Hasenclever and Mitchell again demonstrated the toxicity of live cells given intravenously (26). They further showed that intraperitoneal preinfection with C. albicans or administration of endotoxin, among other treatments, could make mice more resistent to toxicity. This tolerance was associated with the reticuloendothelial system (27).

Braude et al. (7) found that injection of 10^9 viable yeasts or 10^9 autoclaved cells in rabbits gave a febrile response similar to that produced by Gram negative bacterial endotoxin. There was a lag period before temperature increase, the temperature response showed indications of being biphasic, it lasted for about eight hours, and sharp neutropenia immediately followed the challenge. However, tolerance to the febrile activity could not be produced with the fungal preparation as it could with the bacterial system.

Kobayashi and Friedman (44) observed a similar febrile response after intravenous injection of 10^9 heat-killed C. albicans cells. Of three extraction procedures tried, the phenol extract was pyrogenic, but to a lesser degree than killed cells. The cell walls of C.

albicans were also pyrogenic. Neither viable, heat-killed, or phenol extracts of C. albicans could increase dermal reactivity to epinephrine in rabbits, as did endotoxin.

Cutler et al. tested whole C. albicans cells, and fractions of them in systems sensitive to endotoxin (11). Whole cells and cell walls were pyrogenic in rabbits and lethal to actinomycin-D treated mice. Only cell walls were lethal to chick embryos and neither was positive in the limulus lysate assay. Endotoxin was active in all the tests. Alkaline treatment of cells did not destroy toxicity, but did destroy the toxicity of endotoxin. Different extracts of whole cells and cell walls had differing properties. The extracts were mainly carbohydrate with some protein but no toxic fraction had measurable amounts of lipid. Based on the observed differences with bacterial endotoxin, the authors concluded that the toxic substance of C. albicans does not have the same activity as endotoxin despite the noted similarities.

The most complete isolation and characterization of a toxic substance from C. albicans has been reported by Iwata et al. (41). Canditoxin (CT) was isolated from a strain of C. albicans obtained from the spinal fluid of a patient with candidal meningitis. It is a heat-labile, acidic protein with molecular weight about 75,000 daltons. Its LD_{50} for mice is 0.3 ug/g body weight when given intravenously. Infection in mice by the CT-producing strain is significantly enhanced by addition of a sublethal dose of CT. Antibodies to CT, especially IgM antibodies, are active in suppressing infection in mice by the CT-producing strain. Fluorescent labeled anti-CT antibody shows the presence of CT in tissue, especially kidney tissue (41). Here, a strong

case has been made for the existence of a toxin and its role in disease *in vivo*.

Chattaway, Odds, and Barlow (8) examined four strains of *C. albicans*, which were primary isolates from cases of human candidiasis and of proven virulence for mice, for the presence of toxin-like materials. They used a variety of media and growth times and specifically tried to repeat the work of Mourad and Friedman (58), Kobayashi and Friedman (44), Louria, Brayton, and Finkel (50), and Iwata et al. (41). The products were tested for lethality in mice, as was done by the four previous authors. In only one instance were any deaths recorded and these were not repeatable on two separate occasions. Chattaway et al. concluded that toxin production was a strain characteristic because none of their strains produced an active toxin. They further concluded that, because their isolates were virulent for both humans and mice and did not produce a toxic element, "the production of toxin tested in this way bears little relationship to the pathogenicity of this organism."

In few cases has a toxic activity from *C. albicans* been concretely identified. Where it has been found, possession by a large number of strains has not been demonstrated. At present, the significance of toxin in *C. albicans* infection is still ambiguous.

Death From Candida albicans Infection

The cause of death in experimentally-induced *C. albicans* infection in laboratory animals remains controversial. Various authors have suggested that death might be due to embolization (67, 74), toxemia (16, 41, 67), uremia (80, 81), pancreatic damage (86), myocardial damage (1, 60), or hypersensitivity reactions in the lungs (82).

In one study of 25 patients with fatal systemic candidiasis, autopsy records showed that bacterial sepsis, overwhelming candidiasis (involvement of two or more major deep organs), renal failure, and cardiovascular disease were listed as the immediate causes of death (61).

Stovall and Pessin (73) concluded that mechanical occlusion was not the cause of death because larger yeast size (of various species of Candida) did not correlate with ability to cause death with rabbits. Furthermore, some have observed that live cells, but not dead cells at the same dose, are fatal (1). Because the deaths occur fairly soon after challenge, it is unlikely that fungal multiplication occurs to form embolic lesions to an extent which is fatal.

The kidney is by far the organ most frequently and most severely affected by systemic candidiasis in clinical disease in man (49, 59, 61, 69, 83) and in experimental disease in laboratory animals (1, 3, 14, 48, 50, 64, 79). Signficant impairment of renal function as evidenced by elevated BUN has been observed in rabbits at the time of death (80, 81). It was thought that death might be due to uremia.

Significant involvement of the heart has been noted in clinical disease in man (6, 49, 61) and in experimental disease in animals (1, 36, 50, 79). In one study in which tissue population of C. albicans was counted 12 hours after infection, the heart and kidneys had the greatest numbers of viable units (60). In sections of heart tissue taken at this time, there was severe focal myocarditis showing microabscesses and tissue necrosis. A cellular infiltrate was not observed at this time in the kidneys. Elevations in serum lactic dehydrogenase, and glutamic oxalacetic and pyruvic transaminases were also recorded at 12 hours reflecting the pathology observed. A 1.8-fold increase in

BUN over normal controls was thought to represent prerenal azotemia associated with heart failure. These authors imply that serious cardiac damage is involved in fatal C. albicans infection.

Administration of bacterial endotoxin produces hypoglycemia and decreases liver glycogen reserves in the mouse (5). Other biological effects resembling endotoxemia have previously been associated with experimentally induced disseminated candidiasis and acquired clinical disease (6, 7, 39, 40, 44, 49). It has been postulated that C. albicans produces material resembling bacterial endotoxin which may be active in infection (28, 39, 40, 67). Testing for endotoxin's known effects on carbohydrate metabolism during systemic C. albicans infection may yield information as to the possible involvement of a substance similar to bacterial endotoxin.

Blood Urea Nitrogen and Creatinine Clearance

Blood urea is produced in the liver from the deamination of amino acids. It is filtered from the blood, concentrated by the kidneys and is excreted in the urine. Urea is the major form in which surplus nitrogen is eliminated from the body.

BUN measurement is the most widely used test for the screening of kidney function (15). BUN rises when there is excess protein catabolism, decreased hydration of the kidneys, and impairment of renal function. For any increase in BUN, the significance of all three factors must be assessed (29, 46). Because of the tremendous functional reserve of the kidneys and compensatory hypertrophy, BUN does not increase until significant renal damage has occurred (46). Yet BUN will rise before serum creatinine levels increase so in that respect,

it can detect renal damage before the serum creatinine test (29).

Creatinine is a breakdown product of creatine, a compound involved in high energy phosphate storage in muscle. Creatinine is produced in muscle tissue at a constant rate (about 2% turnover per day) and is carried in the bloodstream to the kidneys and then excreted. Blood and urine creatinine levels are very constant in an individual and are not influenced by protein catabolism or the degree of hydration as is BUN (15). Creatinine excretion is determined mainly by the muscle mass (46). Blood creatinine, more so than BUN, may not reflect early renal damage and may be normal even with severe kidney damage (29).

In the kidneys, creatinine is filtered by the glomerulus and, under normal conditions, no tubular secretion or reabsorbtion occurs. Its clearance is thus a good measure of the glomerular filtration rate (GFR) (15, 29, 46). If the blood creatinine is elevated, however, some tubular secretion occurs in which case creatinine clearance is not as accurate a measure of the GFR (29). With renal damage, blood creatinine tend to decrease. Thus the creatinine clearance rate tends to decrease.

METHODS AND MATERIALS

Mice

Female mice (HA/ICR) weighing 24-28 g were obtained from Spartan Research Animals, Haslett, Michigan. They were housed six animals per cage and, under normal conditions, water and food (Wayne Lab-Blox, Allied Mills, Inc., Chicago, Illinois) were available ad libitum. Fasting animals had no food but had access to water. Bilaterally nephrectomized mice were given 0.85% saline instead of water.

Cultivation of Fungi

A strain of C. albicans previously isolated from a case of vaginal candidiasis at Olin Health Center, Michigan State University, was identified by Dr. A. L. Rogers and supplied by Dr. R. T. Sawyer for use in this study. Stock cultures were maintained on Sabouraud dextrose agar (SDA, Difco Laboratories, Detroit, Michigan) slants at room temperature. To prepare innocula, a transfer was made from a slant into 100 ml of tryptic soy broth (Difco) supplemented with 4% D-glucose (Fisher Scientific Co., Fair Lawn, New Jersey) in a 250 ml culture flask. This was incubated for 12-14 hours with agitation at 37 C. Cells were harvested by centrifugation at 3000 rpm for 10 minutes (Phillips-Drucker tabletop centrifuge, Asotria, Oregon) and then washed three times with sterile 0.85% saline. A cell count was obtained using a hemacytometer. This was confirmed by pour plates of tenfold dilutions on SDA. The proper

dilution was made with 0.85% saline to reach the desired concentration for injection. Heat-killed C. albicans cells were prepared by incubation in a 56 C water bath for one hour. Injections were given with a 1 ml disposable syringe and a 27-gauge needle (Becton-Dickenson, Rutherford, New Jersey) intravenously in a tail vein, or intraperitoneally.

Tissue Distribution Studies

To determine the number of viable organisms in various tissues of infected mice, the organs were aseptically removed, weighed, and placed in separate, sterile glass homogenizing tubes. Sterile 0.85% saline was added so the total volume in each tube was 10 ml. The samples were homogenized using a Tri-R Stir-R tissue homogenizer (model S63C, Tri-R Instruments, Inc., Rockville Centre, New York) and pour plates of the appropriate dilutions in saline were made on SDA. The plates were incubated at 37 C for 36-48 hours and counted.

Blood Urea Nitrogen Assay

BUN was determined using the method of Coulombe and Favreau (10). Briefly, a heparinized syringe with a blunted 18-gauge needle was used to collect 0.1 ml whole blood from the retro-orbital plexus of each mouse. For the reagent blank 0.1 ml deionized water (dHOH) was used and for preparation of a standard curve 0.1 ml urea (Mallinkrodt Inc., St. Louis, Missouri) standards ranging from 10 to 200 mg% (prepared by appropriate dilution of a stock urea standard containing 2 g/100 ml) were used. Tungstic-acid reagent was prepared by diluting 10% (w/v) sodium tungstate (Mallinkrodt) 1:10 with a solution containing 2.32 ml conc. H_2SO_4 per liter dHOH. Reagent A was prepared by adding two parts of a solution containing 0.6 gm 2,3-butane-dione monoxine (Sigma

Chemical Co., St. Louis, Missouri) and 0.3 g thiosemicarbazide (Sigma) per 100 ml dHOH to ten parts 60% conc. phosphoric acid. The volume of sample or standard was added to 0.9 ml tungstic-acid reagent and allowed to stand at room temperature for five minutes. After centrifugation at 3000 rpm for five minutes (Phillips-Drucker tabletop centrifuge), 0.2 ml supernatant was added to 5 ml Reagent A and mixed vigorously. This was heated for 20 minutes in a boiling water bath and then cooled in running tap water. The red color which developed was read spectrophotometrically at 530 nm on a Gilford 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The mg% BUN was read from the standard curve.

Creatinine Assay and Creatinine Clearance

Endogenous creatinine clearance was determined by collecting urine and blood from the same animal and measuring the creatinine concentration in each. For urine collection, mice were housed individually in metabolism cages for 12 hours (10PM to 10AM). They were fasted during the period of urine collection but not before. At the end of the 12 hour period, a heparinized syringe was used as above to collect 0.5 ml whole blood.

Blood and urine creatinine were assayed by the method of Faulkner and King (15) with slight modification. To 0.5 ml heparinized whole blood in a glass tube was added 0.5 ml 5% sodium tungstate, 0.5 ml 0.66 N sulphuric acid, and 0.5 ml dHOH. The sample was then centrifuged at 3000 rpm for ten minutes. Urine was prepared by centrifuging out fecal material, drawing the sample into a pipette to measure volume, and diluting 1:40 with dHOH. Creatinine standards were prepared by appropriate dilution with dHOH of a stock solution containing 0.143 g

creatinine sulphate in 100 ml of 0.1 N HCl (1 mg/ml creatinine). One ml of deproteinized whole blood supernatant, 1.0 ml diluted urine, 1.0 ml diluted standard, or 1.0 ml dHOH (reagent blank) was put in each of a series of glass tubes containing 1.0 ml dHOH. To each tube was added 0.5 ml 0.04 M picric acid (Baker Chemical Co., Phillipsburg, New Jersey) and 0.5 ml 0.75 N NaOH, mixing after each addition. The optical density at 500 nm was determined after incubation at room temperature for 20 minutes, using a Gilford 240 spectrophotometer. The standard curve gives the blood creatinine concentration in mg/100 ml directly and gives the urine creatinine concentration in mg/100 ml when multiplied by a factor of ten.

Creatinine clearance was calculated in ml plasma cleared per minute by the formula U·V/P where U=urine creatinine concentration (mg%), V=urine volume (ml), and P=blood creatinine concentration (mg%).

Liver Glycogen Assay

Liver glycogen was assayed by the method of Kemp and Kits van Heijningen (43). Mice were killed by cervical dislocation and a piece of liver tissue weighing 30-50 mg was excised. This was added to 5 ml deproteinizing solution (5 gm trichloroacetic acid and 100 mg Ag_2SO_4 diluted to 100 ml with dHOH) in a glass homogenizing tube. The reagent blank contained 4 ml deproteinizing solution and 1 ml dHOH and standards contained 4 ml deproteinizing solution and 1 ml of the appropriate dilution of a stock standard containing 16 mg/ml dextrose. Tissue was homogenized using a Tri-R Stir-R tissue homogenizer. The liver homogenates were placed in a boiling water bath for 15 minutes, cooled in tap water, and centrifuged at 3000 rpm for five minutes (model PR1, International Equipment Co., Boston, Massachusetts). One ml of the

supernatant was added to 3 ml conc. sulphuric acid. This was mixed vigorously and then heated in a boiling water bath for 6.5 minutes. The red color was read spectrophotometrically at 520 nm (Coleman 111 Hitachi Perkin-Elmer, Arthur C. Thomas Co., Philadelphia, Pennsylvania). Milligrams of liver glycogen were read from the standard curve. Mg glycogen/mg of liver tissue excised x 100 gives the percent liver glycogen.

Blood Glucose Assay

Initially, blood glucose was determined by the Glucostat method (Worthington Biochemical, Freehold, New Jersey). One-tenth ml heparinized whole blood was collected from the retro-orbital plexus of each mouse as previously described. This was diluted to 2.0 ml in dHOH. For the reagent blank, 2.0 ml dHOH was used and for standards, 0.1 ml of the appropriate standard solution, prepared from a 200 mg% dextrose stock standard, was added to 1.9 ml dHOH. To each tube was added 1.0 ml of 1.8% $Ba(OH)_2$ and 1.0 ml of 2.0% $ZnSO_4$ for deproteinization. (The two solutions were prepared and then adjusted so that equal volumes neutralized each other to a phenolphthalien endpoint.) This was centrifuged at 3000 rpm for five minutes (Phillips-Drucker tabletop centrifuge) and 2.0 ml of supernatant from each sample was pipetted into separate test tubes. At 15 second intervals, 2.0 ml of reconstituted Glucostat reagent was added to each tube. After ten minutes incubation at room temperature, one drop of 4 N HCl was added to each tube at 15 second intervals to stop the reaction. The yellow color which developed was read at 420 nm using a Perkin-Elmer spectrophotometer.

During the course of this study, the commercial reagent and procedure were changed by the manufacturer. After this point, a modificaton of the Glucostat method (Worthington) was used. One-tenth ml heparinized whole blood was added to 0.9 ml dHOH. Standards and the reagent blank also had a final volume of 1.0 ml. Deproteinization was carried out as before. Then 1.0 ml supernatant was added to 1.0 ml of the reconstituted Glucostat reagent prewarmed in a 37 C water bath. After 30 minutes incubation at 37 C, the tubes were centrifuged for five minutes at 3000 rpm to remove residual turbidity and the optical density was determined at 500 nm using a Gilford 240 spectrophotometer.

Appropriate standard curves were prepared for each procedure, from which the mg glucose/100 ml whole blood was read. Data are expressed as mg% blood glucose.

Nephrectomy Procedures

Mice were anaesthetized by subcutaneous injection of 0.25 mg sodium pentabarbitol (Butler Co., Columbus, Ohio) followed by light etherization. They were restrained on a board with tape, the hair was shaved from the back, and a solution of 2% tincture of iodine (Baker) was swabbed on the back to disinfect. A 3 cm incision was made through the skin over the backbone using small surgical scissors. The skin was pulled to the left and a 1.5 cm incision was made through the muscle layer parallel to and 0.5 cm to the side of the spinal column. The kidney was then excised. For a bilateral nephrectomy the same procedure was followed on the right side. The muscle layers were not sutured. A curved surgical needle and 000 cutgut suture were used to make three or four sutures to close the skin incision. Unilaterally nephrectomized mice were kept for an experimental period of one

month. Bilaterally nephrectomized mice were kept until the time of their death.

Endotoxin

Lipopolysaccharide prepared by phenol-water extraction from Salmonella typhimurium was purchased from Difco Laboratories, Detroit, Michigan. It was suspended in pyrogen-free saline to a concentration of 5.0 mg/ml and 1.0 ml aliquots were stored at -20 C until used. For injection, the aliquot was thawed and diluted further in saline to the desired concentration.

Statistics

Statistical significance was determined by using the White Rank Order Test (78). Mean, Standard deviation, and standard error were calculated according to conventional statistical methods.

RESULTS

Survival of Mice Infected with Candida albicans

Groups of female mice weighing 24-28 g were given either 1.0 x 10^6 or 4.5 x 10^6 cfu C. albicans intravenously. Survival was recorded until all animals had succumbed. In mice receiving 1.0 x 10^6 cfu the first deaths occurred on day one but some mice survived as long as 16 days (Figure 1). For this group, the mean survival time was 8.8 days. All mice receiving 4.5 x 10^6 cfu died between 8 and 12 hours after infection. The mean survival time was 9.0 hours.

It is evident from the survival data that the two slightly different challenge doses of C. albicans result in very different patterns of morbidity and mortality. Some pathophysiologic differences in these two responses are described below.

Tissue Populations in Infected Mice Given Approximately 1.0 x 10^6 Candida albicans

The distribution of 1.0 x 10^6 C. albicans among the brain, heart, kidney, liver, lungs, and spleen was determined at 30 minutes after injection and at the time of death. The results are shown in Table 1.

The data show that in four of the six organs studied, (liver, spleen, kidneys and brain), the number of organisms recovered increased from 30 minutes to near the time of death. The kidney shows the most dramatic increase (over 1000X). In two organs (lungs and heart), the

Figure 1. Survival of mice infected with C. albicans.
● = challenge dose of 1.0 x 10^6 cfu, iv.
■ = challenge dose of 4.5 x 10^6 cfu, iv.

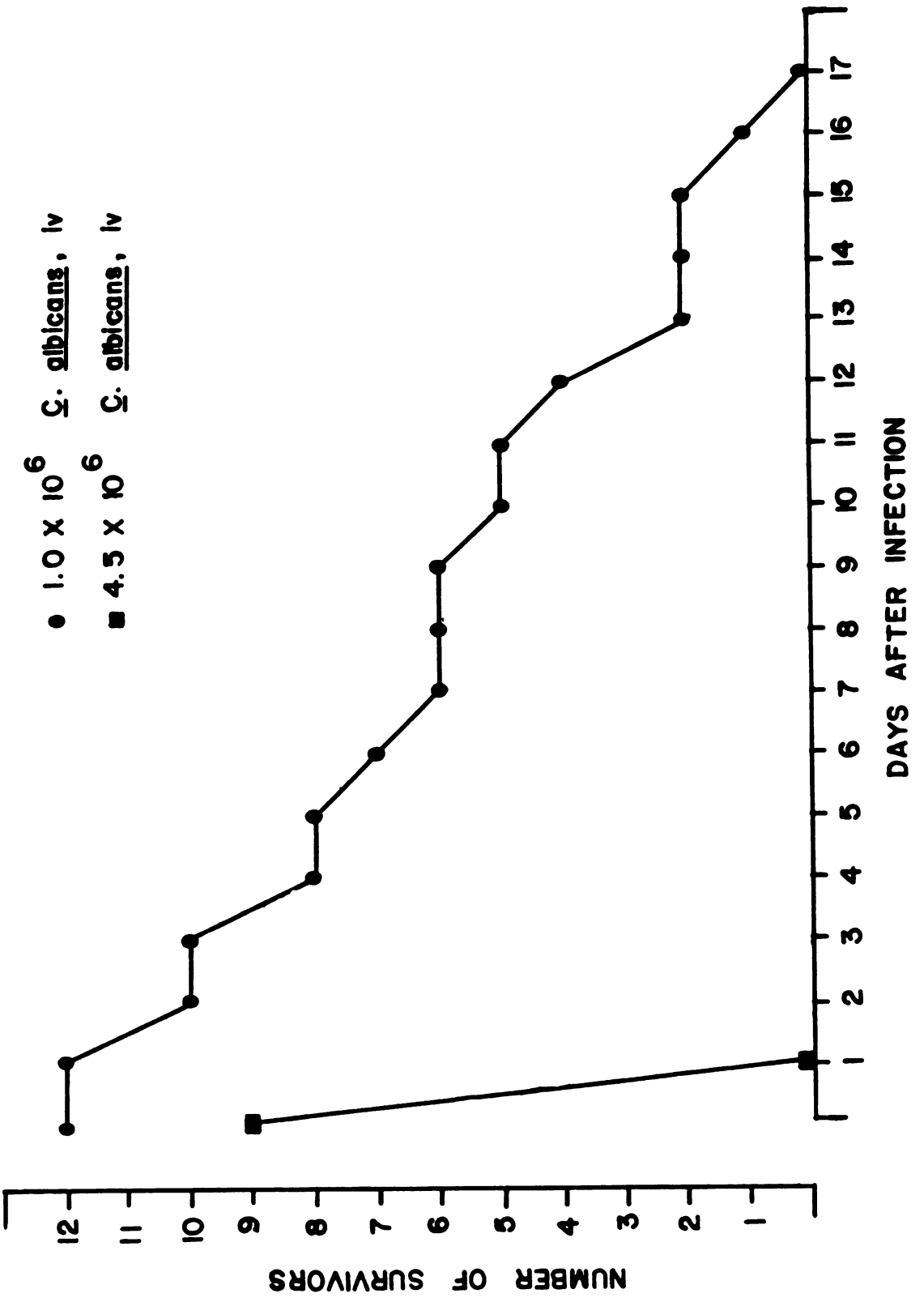
● 1.0 X 10^6 C. albicans, iv
■ 4.5 X 10^6 C. albicans, iv
NUMBER OF SURVIVORS
12
11
10
9
8
7
6
5
4
3
2
1
DAYS AFTER INFECTION
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17

FIGURE 1

Table 1. Tissue populations in infected mice given approximately 1.0 x 10^6 C. albicans.

	30 minutes after challenge (# cfu/organ)		at death (# cfu/organ)	
Liver	7.5×10^4[a]	(2.6)[b]	1.4×10^5	(0.9)
Lungs	2.3×10^5	(1.3)	4.8×10^4	(7.7)
Spleen	1.3×10^3	(1.2)	2.7×10^3	(3.3)
Kidneys	1.8×10^4	(0.6)	2.7×10^7	(2.6)
Heart	2.4×10^4	(0.9)	2.3×10^3	(5.6)
Brain	1.0×10^4	(0.6)	3.1×10^5	(3.8)

[a]mean of at least six mice.

[b]number in parentheses is standard deviation.

number of organisms decreased from 30 minutes after infection to the time of death.

At 30 minutes, approximately 5% of the organisms recovered from the major host organs was localized in the kidneys while at the time of death, over 98% of the organisms recovered for the six organs were isolated from the kidneys (not shown in table). In all other organs, the percentage of the total recovery from the organs decreased from 30 minutes after infection to the time of death. The percentage in the liver decreased from 20.9% to 0.5%, in the lungs from 64.2% to 0.2%, in the spleen from 0.4% to 0.01%, in the heart from 6.7% to 0.01%, and in the brain from 2.8% to 1.1%.

BUN and Blood Creatinine in Normal, Infected, and Nephrectomized Mice

BUN was measured daily on mice infected with 1.0×10^6 *C. albicans* until all animals had died. Normal mice were used to determine whether multiple bleedings had any effect on the BUN. The results are shown in Figures 2a and 2b.

Figure 2a shows BUN changes versus days after infection. In normal mice, BUN remained relatively constant throughout the experimental period. In infected mice, the curve is quite chaotic with large standard deviations and no definite pattern. As noted above, large fluctuations in the time of death within the experimental group occurred. On any given day, the mean BUN expressed in Figure 2a is the average of all animals still alive, whether they are very near to or very far from the eventual time of death.

Figure 2b shows BUN in infected mice on days before death. This figure clearly shows that BUN in infected mice starts to rise about four days before death and increases to very high levels as death approaches. It reaches its highest peak on the day of death.

Table 2 compares BUN and blood creatinine levels in experimentally manipulated mice. BUN levels are approximately 50.0 mg% in normal mice and are elevated to more than 450 mg% (> 9X) at the time of death.

Unilateral nephrectomy does not significantly increase BUN levels and such animals survive on routine laboratory maintenance for at least one month. Bilaterally nephrectomized mice live for 30 hours at most. BUN increases very rapidly and is approximately the same value as for infected mice near the time of death. Similar changes are noted in blood creatinine levels. Normal and unilaterally

Figure 2a. BUN vs. time in normal and infected mice.
● = normal mice; ■ = infected mice
(1.0 x 10^6 cfu C. albicans, iv).

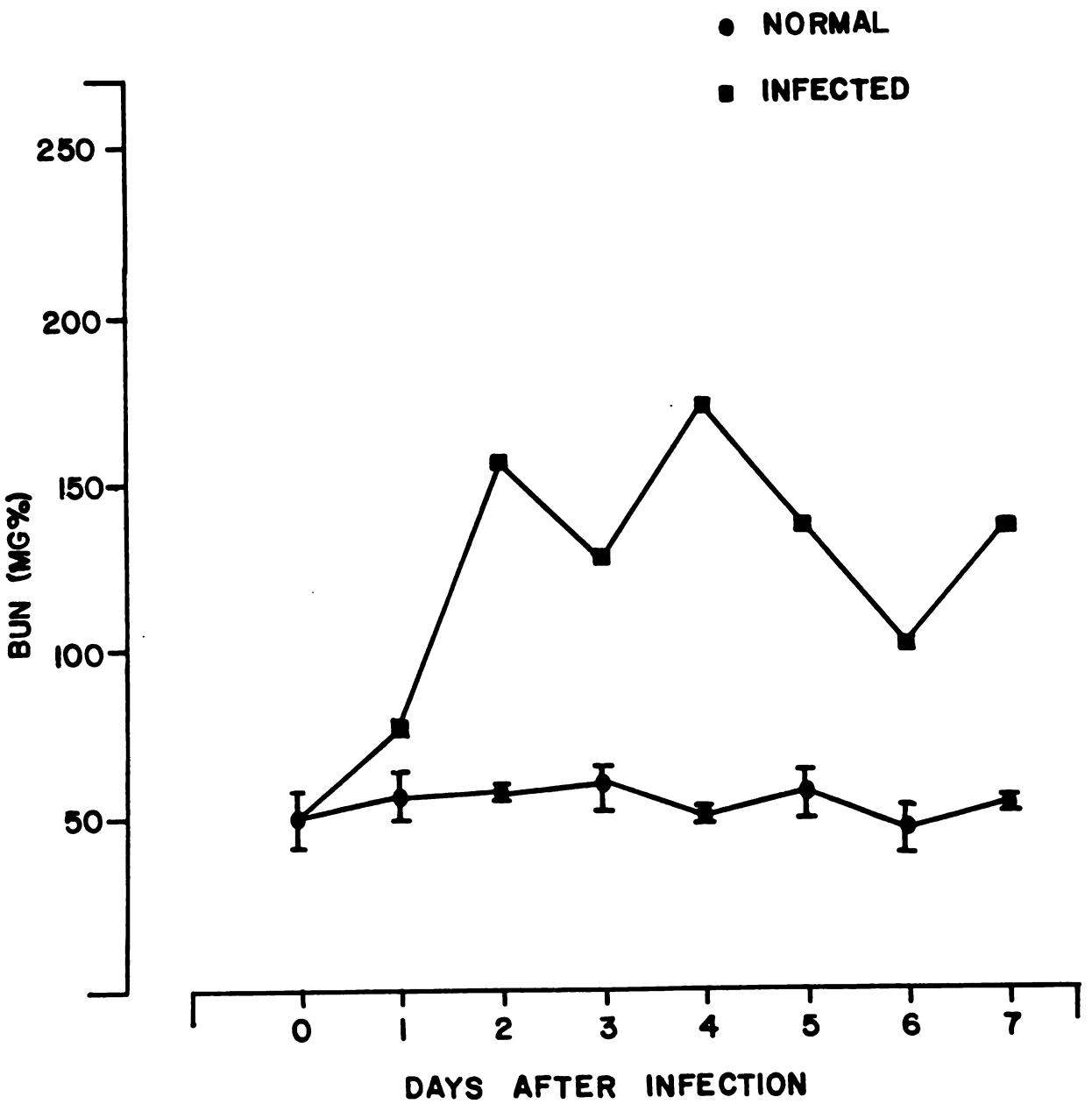
NORMAL
INFECTED
250
200
150
100
50
BUN (MG%)
0
1
2
3
4
5
6
7
DAYS AFTER INFECTION

FIGURE 2a

Figure 2b. BUN in infected mice (1.0×10^6 cfu) on successive days before death. Day 0 = day on which death occurred.

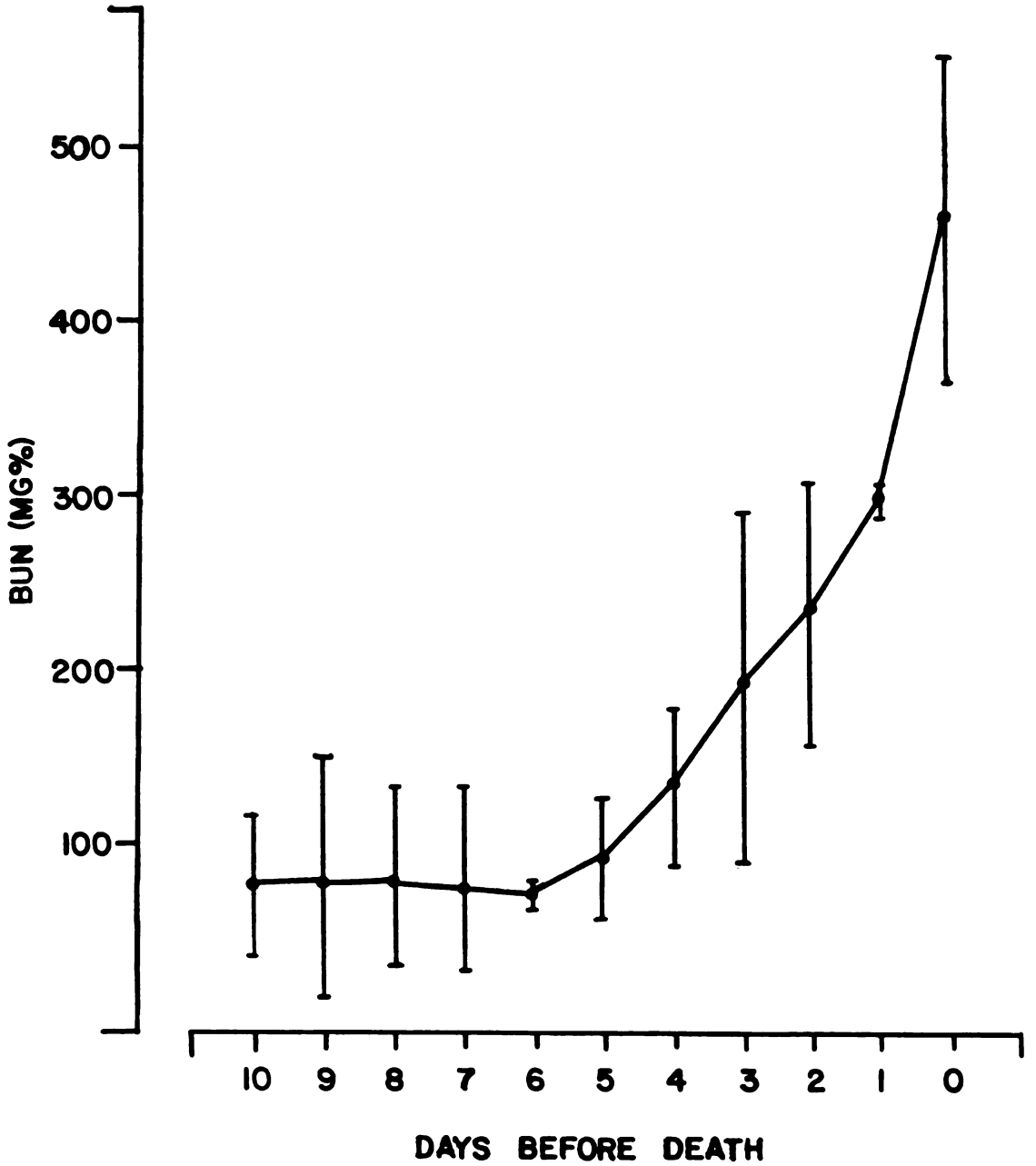

FIGURE 2b

Table 2. BUN and blood creatinine values for normal, infected, and nephrectomized mice.

Treatment	BUN (mg%)	Blood creatinine (mg%)
None	50.0± 8.7 (12)[a]	0.48±0.12 (18)
Infected (1.0×10^6 cfu)[b]	463.8±48.5 (7)	1.79±0.84 (6)
Unilateral nephrectomy[c]	53.8±12.2 (6)	0.46±0.08 (5)
Bilateral nephrectomy[b]	427.6±15.3 (3)	2.57±0.29 (3)

[a]Number in parentheses indicates number of animals in group.

[b]Assays were done at the time of death of the mouse.

[c]Value shown is the mean of at least five separate determinations made over one month following surgery.

nephrectomized mice have essentially the same levels of blood creatinine whereas infected and bilaterally nephrectomized mice have values three to six times higher than normal mice.

Creatinine Clearance in Normal and Infected Mice

The creatinine clearance rate for normal and infected mice was determined in three separate experiments with six animals in each group. The results are given in Table 3. Normal mice had a creatinine clearance rate of about 89.1 ml plasma/minute while infected mice cleared only about 44.1 ml plasma/minute showing that by four days after infection, the creatinine clearance rate had decreased to approximately half that of normal mice.

Table 3. Creatinine clearance in normal and infected mice.

	Creatinine clearance[b] (ml plasma cleared/min)
Normal mice	89.1±14.7[c] (18)[e]
Infected mice[a]	44.1± 3.9[d] (18)

[a]Mice were given 1.0 x 10^6 C. albicans iv four days previously.

[b]Mean ± standard error.

c vs. d significantly different at .001 level.

[e]Total number of animals assayed.

Liver Glycogen and Blood Glucose Changes in Normal and Infected Mice

Table 4 shows liver glycogen and blood glucose values for normal and infected mice after 48 hours and very near the time of death. At both times, liver glycogen reserves were lower in infected mice than in normal mice, yet even very near the time of death, liver glycogen was not depleted. Blood glucose levels of infected mice were not significantly different from normal values at any time points tested. Comparisons were also made at 4, 8, 12, 24, and 36 hours, but only 48 hours and at the time of death are reported.

Serum Biochemistries on Mice Infected With 4.5 x 10^6 cfu Candida albicans

Liver glycogen, blood glucose, and BUN changes were measured in mice after infection with this higher challenge dose (Table 5). Liver glycogen steadily decreases with time after infection, yet even at eight hours, the liver glycogen reserves of infected mice were not

Table 4. Liver glycogen[b] and blood glucose changes in normal and infected mice.

	Normal mice	Infected mice[a]	
		48 hr post-challenge	At death
Liver glycogen (%)	4.99[c]± 1.16	1.44± 1.31	1.12± 1.43
Blood glucose (mg%)	103.0 ±10.0[d]	79.8 ±23.9[d]	82.5 ±29.4[d]

[a]Infected mice received 1.0 x 10^6 cfu C. albicans iv.

[b]Values represent mean ± standard deviation of at least six mice.

[c]All values for this mean only assayed at 10 AM.

[d]Not significantly different.

Table 5. Serum biochemistries on mice infected with 4.5 x 10^6 cfu C. albicans.[a]

Time post-challenge (hr)	Liver glycogen (%)	Blood glucose (mg%)	BUN (mg%)
0[b]	4.99±1.16	103.0±10.0[c]	50.0± 8.7[d]
1	3.68±1.12	112.2±10.5	47.5± 8.1
4	1.67±0.97	116.7±29.0	39.5± 6.2
8	0.87±0.68	132.6±30.8[c]	54.1±14.0[d]

[a]Mean ± standard deviation of at least six mice.

[b]Values for normal mice.

[c]Significantly different at .05 level.

[d]Not significantly different.

significantly different from those of saline control mice which show a circadian drop in liver glycogen during this time of day (data not shown). Blood glucose increases after infection and hyperglycemia is evident as the time of death approaches. The BUN level of infected mice near death is within a normal range.

Serum Biochemistries on Mice Given Saline, Heat-killed Candida albicans, or Endotoxin

Mice were infected intraperitoneally with either 4.5×10^6 or 1.0×10^9 heat-killed C. albicans in 0.7 ml saline. S. typhimurium endotoxin (150 ug/mouse in 0.3 ml saline) was injected intraperitoneally. Mice given 0.5 ml saline served as controls. Values for liver glycogen, blood glucose, and BUN were obtained 24 hours after injection and are shown in Table 6. The lower dose of heat-killed C. albicans gave values for the three assays which were similar to those for saline controls. Treatment with 1.0×10^9 C. albicans cells depleted liver glycogen and caused hypoglycemia, but did not affect BUN. Endotoxin decreased liver glycogen to very low levels and caused hypoglycemia. Unlike the higher yeast dose, endotoxin caused a marked elevation of BUN over saline controls.

Table 6. Serum biochemistries on mice given saline, heat-killed *C. albicans*, or endotoxin.[a]

Treatment	Liver glycogen (%)	Blood glucose (mg%)	BUN (mg%)
Saline	3.60±1.83	90.6±12.3	52.1±14.6
C. albicans (4.5×10^6 heat-killed cells, ip)	4.11±0.53	103.3± 7.1	31.9± 5.3
C. albicans (1.0×10^9 heat-killed cells, ip)	0.04±0.05	34.3± 7.2	31.1±12.7
Endotoxin (150 ug, ip)	0.22±0.14	51.8±14.8	106.5±44.9

[a]Values shown represent mean ± 1 standard deviation and were obtained 24 hours after ip injection.

DISCUSSION

The survival data shown in Figure 1 confirms the previously reported finding (58) that a threshold dose for C. albicans infection exists below which a progressively fatal infection occurs and above which rapid death ensues. The differing outcomes of challenge with relatively small differences in dose suggests that different mechanisms may be involved in death.

Death with the lower dose probably results from progressive multiplication of the fungus within host tissues. Tissue populations were counted in six major host organs shortly after challenge and near the time of death. Table 1 shows that while a relatively small percentage of the original innoculum localizes in the kidneys, almost all of the organisms recovered from the major organs (approximately 98%) are found in the kidneys near the time of death. This represents over a 1000-fold increase in the number of organisms recovered from this organ. While some organs show an increase in the number of organisms, none are as substantial as that seen in the kidney.

Previous studies suggest that the challenge dose is destroyed in all organs except the kidney, while the challenge dose multiplies in the kidneys (14, 48, 50). If this is so, then the organisms recovered from the liver, spleen, brain, lungs, and heart may have disseminated from the kidneys. The data in Table 1 do not prove that the organisms recovered near the time of death are descended from those recovered

after 30 minutes. Nor do the data in Table 1 express total recovery from the host. Fungi are present in parts of the host not counted such as the carcass (unpublished data); furthermore, clumping of organisms could create artifacts in viable counts compared to their actual values. Nonetheless, the conclusion from Table 1 that a very large number of organisms burden the kidneys near the time of death is inescapable.

The possibility that the large tissue burden in the kidneys is responsible for serious alteration of renal function was investigated by assaying BUN (Figure 2b), blood creatinine (Table 2), and creatinine clearance (Table 3). Mice receiving 1.0×10^6 *C. albicans* showed increases in BUN with time to very high levels (Figure 2b, Table 2). Our data confirms and extends the observations made by Winner (80) that BUN values for infected rabbits at death range from 110-410 mg%. By expressing the data with respect to time of death rather than time post-infection we have removed much of the extensive variability seen in previously published studies and can definitively conclude that BUN rises to fatal levels at the time of death. The regularity in the quantitative increase in BUN could almost be used to assay for severity of *in vivo* infection and also predict time to death in untreated mice.

Similarly, blood creatinine values are elevated in infected mice (Table 2). For both BUN and blood creatinine, the increases observed are comparable to those occurring for mice whose kidneys have been surgically removed. It is interesting to note that unilaterally nephrectomized mice, which have half of their renal function removed, have normal BUN and blood creatinine values (Table 2). Evidently the tremendous renal function reserve and compensatory hypertrophy can made up for the loss in renal functioning capacity. This lends further

creedence to the notion that serious renal damage has occurred in infected mice, whose BUN and blood creatinine values do rise significantly above normal.

Both BUN and blood creatinine values are influenced by factors other than renal status (29, 46). Also, both parameters are slow to indicate renal impairment and may remain normal even in the face of significant renal damage (29, 46). Creatinine clearance measures the glomerular filtration rate and represents a more sensitive measure of kidney function (15). By showing a decreased creatinine clearance rate at four days after infection, Table 3 indicates that the elevations in BUN and blood creatinine do indeed reflect impairment of renal function.

Altogether, the data underline the serious involvement of the kidneys in C. albicans infection. It can be concluded that at the lower challenge dose (1.0×10^6 cfu), mice are dying from renal failure associated with a serious renal fungal infection.

It seems unlikely that death from the higher dose (4.5×10^6 cfu) results from any kind of infectious process because of how quickly it follows challenge. In this case, death seems more like a toxic rather than an infectious process.

This possibility was investigated by studying the metabolic parameters of liver glycogen, blood glucose, and BUN in mice infected with 4.5×10^6 cfu. For comparative purposes, these parameters were also measured in mice given 1.0×10^6 cfu. That endotoxin depletes carbohydrate and elevates BUN is well established (5).

The data in Table 5 show that metabolic status near the time of death is different for the two experimental groups. BUN is normal and hyperglycemia is evident in mice given 4.5×10^6 cfu while mice

receiving 1.0 x 10^6 do not exhibit such changes (Tables 2 and 4).

It has been suggested that endotoxin-like material may be associated with C. albicans and various biological activities similar to endotoxemia have been demonstrated in animals receiving preparations or extracts of C. albicans cells (7, 11, 26, 28, 44, 58, 67). For this reason, the metabolic changes induced by endotoxin were compared to those for mice receiving either 4.5 x 10^6 or 1.0 x 10^9 heat-killed C. albicans. The latter dose was included because previous work has indicated that a pyrogenic response closely resembling endotoxin is associated with this dose of C. albicans (44). Both endotoxin and 1.0 x 10^9 heat-killed C. albicans produced severe hypoglycemia by 24 hours but, unlike endotoxin, heat-killed C. albicans cells did not elevate BUN (Table 6). It is doubtful that this large amount of fungal material would ever occur with naturally acquired C. albicans infection in mice. By contrast, 4.5 x 10^6 cells which produce rapidly fatal disease (Figure 1) do not produce abnormalities in liver glycogen, blood glucose, or BUN.

Although it was not determined here if fungal multiplication occurred before death in mice receiving 4.5 x 10^6 cfu C. albicans, Louria et al. (48) reported that C. albicans populations in liver, spleen, heart, lungs, and brain did not change significantly in the first 24 hours after infection with 5 x 10^6 viable cells. That the values for the metabolic parameters studied were different in mice near death from the two C. albicans challenge doses (Tables 2 and 5) would also implicate a different cause of death for each dose.

The events leading to death after challenge with 4.5×10^6 cfu are not well understood. It is not known if a certain critical level of a toxic entity (which is present with 4.5×10^6 cfu and possibly is reached with fungal multiplication after challenge with 1.0×10^6 cfu) precipitates death. If a toxin is present in the C. albicans preparations, it does not closely resemble bacterial endotoxin. Friedman et al. (11) arrived at a similar conclusion by monitoring different biological parameters. Nor does it resemble classical bacterial exotoxins. Yet toxicity remains a demonstrable phenomenon.

In summary, mice given 1.0×10^6 cfu C. albicans manifest a progressive fungal infection resulting in serious renal involvement and severe impairment of renal function leading to death. Mice given 4.5×10^6 cfu C. albicans die much sooner after challenge. If a toxin is involved in death at this dose, it does not closely resemble bacterial endotoxin.

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