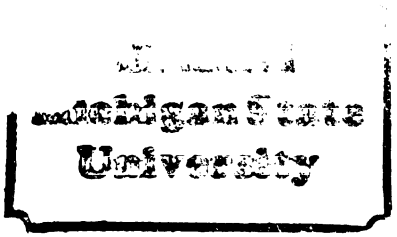

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MURINE RESPONSES TO CANDIDA ALBICANS: IN VIVO FOOTPAD AND IN VITRO PHAGOCYTIC STUDIES

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JAMES MICHAEL VESELENAK

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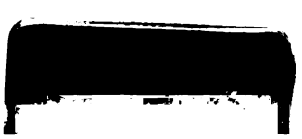

I AM I AM I AM superman
and I know whats happening.
I AM I AM I AM SUPERMAN
AND I CAN DO ANYTHING.

R.E.M.

WHAT THE FUCK

MURINE RESPONSES TO *CANDIDA ALBICANS*: *IN VIVO* FOOTPAD AND *IN VITRO* PHAGOCYTIC STUDIES

By

James Michael Veselenak

A DISSERTATION

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ABSTRACT

MURINE RESPONSES TO CANDIDA ALBICANS: IN VIVO FOOTPAD AND IN VITRO PHAGOCYTOSIS STUDIES

by

James Michael Veselenak

In vivo footpad responses to injected Candida albicans were characterized and compared in non-sensitized, sensitized and cylophosphamide (CY) pretreated mice. Non-sensitized mice reacted to an injection of live yeast cells with a maximal 4 h footpad swelling response. Greater than 99% of the injected yeast cells were killed by 72 h. Mice were sensitized by prior subcutaneous injections of heat-killed yeast cells and showed specific footpad responses as well as antibodies to the antigen. Sensitized mice were not immune to systemic infection when compared to non-sensitized mice. Greater footpad responses were seen in sensitized mice after injection of live yeasts although the rate of killing was no different than in non-sensitized mice. Cyclophosphamide pretreatment resulted in a severe granulocytopenia. These animals were more susceptable to systemic candidosis. Footpad swelling responses were similar to those of non-pretreated animals, but the ability to control the localized infection was impaired.

Histopathological examination of footpad sections after infection revealed an early infiltration which was exclusively granulocytic in non-sensitized mice while sensitized mice showed a predominantly

granulocytic infiltrate although later a mononuclear component was evident. Responses seen in CY pretreated mice were similar in cellular makeup but less intense than in non-drug treated mice. Fungal proliferation within necrotic areas was seen.

Polymorphonuclear neutrophils were used in the *in vitro* phagocytosis and candidacidal assays. Approximately 50% of the phagocytosed yeasts were inhibited in germ tube formation while CY pretreatment resulted in 29% and 16% germ tube inhibition for the 100 mg/kg and 200 mg/kg groups, respectively. Yeast cells and PMN incubated for 2 h showed approximately 43% of the yeasts killed in the non-CY groups while up to 90% were still viable in the 200 mg/kg groups. No effect was seen with PMN from non-drug treated mice when incubated in various concentrations of CY.

Cumulatively these data suggest that non-specific host defense mechanisms are primarily responsible for the control of infection with *C. albicans*. Any alteration of this system resulting from the use of cytotoxic or immunosuppressive agents will predispose the host to infection by this organism. In addition, immunological hypersensitivity to this yeast does not necessarily imply immunity to infection.

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DEDICATION

To my parents, Michael and Rita Veselenak, for understanding why I wanted to go to school for so long.

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INTRODUCTION

Resistance to infection by the myriad of microorganisms in the environment is based upon non-specific factors and specifically acquired immunity which frequently operates in concert with these non-specific factors, thereby greatly increasing their effectiveness (Roitt, 1974).

Infection caused by the opportunistic yeasts, especially Candida albicans, are becoming more common due to advances in antimicrobial and chemotherapeutic agents. This yeast is a commensal in the intestinal tract of a significant portion of the population and therefore any alteration of the host may predispose the host to infection by this organism. Thus, a better understanding of the basic host-microbe interaction is necessary.

Penetration of a foreign microorganism into the tissues of the body results in an inflammatory response. Phagocytic cells migrate from the bloodstream to the site of the injury. This is a non-specific process, in that no previous exposure to the microbe is necessary for proper function. These cells phagocytose the invading organism and either the infection is limited and resolved or the microbe escapes killing and the infection proceeds, possibly to other body sites. The polymorphonuclear leukocyte (PMN) is a short-lived, highly phagocytic cell that comprises a major portion of this non-specific defense system. These cells are usually the first phagocytic cells to migrate to the area of infection or injury.

The immune response is a remarkably versatile adaptive mechanism in which animals form specifically reactive proteins and cells in

response to a variety of compounds, from macromolecules to whole organisms. It constitutes a principle means of defense not only against infection by pathogenic microorganisms and viruses, but probably also against host cells which transform into cancer cells. Prior exposure, either natural or active, with a particular agent is usually necessary to induce specific immune responses directed to the agent.

An *in vitro* mouse footpad assay model for quantitatively studying intralesional numbers of viable yeast cells was developed. Using this model along with histopathological observations, the intralesional events after infection can be followed. *In vitro* studies of the phagocytic and killing properties of the PMN showed the important role of this cell in host defense to *Candida*.

The primary objective of this study is to characterize the host response to a localized *C. albicans* infection. Sensitization as well as debilitation of the host prior to challenge were used to better understand the host-parasite relationship regarding this unique microorganism.

LITERATURE REVIEW

Candida albicans

The first recorded account of disease caused by Candida albicans was that of Hippocrates (Rippon, 1974). The aphthae (white patches) in debilitated patients were described in his "Epidemics". The term "thrush" probably arose as an ancient Scandinavian or Anglo-Saxon word (Rippon, 1974; Odds, 1979). The first concise descriptions of oral and gastrointestinal candidosis were by Rosen von Rosenstein in 1771 and Underwood in 1784 (Odds, 1979). Candida infections of virtually every tissue in the human body have been reported. Although the most common manifestations of candidosis are superficial lesions of mucocutaneous surfaces, the incidence and severity of systemic disease is increasing due to advances in modern medicine and the use of antibiotics (Seelig, 1966), immunosuppressive and cytotoxic therapies (Green, 1968), and intravenous infusions (Klainer and Beisel, 1969).

The taxonomic position of the genus Candida was established by the Eighth Botanical Congress at Paris in 1954 (Rippon, 1974). Members of this genus belong to the Form-class Deuteromycetes, Form-subclass Blastomycetidae, and Form-order Cryptococcales (Alexopoulos and Mims, 1979). Despite the validity of this genus, the terms "Monilia" and "moniliasis" still appear regularly in present-day textbooks and learned journals.

The ecology of C. albicans is the alimentary tract of mammals and birds (Rippon, 1974) and all such species are susceptible to invasion

by this fungus (Smitten, 1967). Rarely, has C. albicans been isolated from the soil, but in these instances it probably represents fecal contamination.

In the human, carriage rates for C. albicans vary with the many reports published. In the epidemiological studies reported, variables such as patient selection, methods of obtaining specimens, identification procedures, and geographical location contribute to the wide range of results obtained. Recovery of C. albicans from the oral cavity, although ranging from 2 to 37%, show that in a majority of studies the carrier rate is less than 20% for normal individuals (Schmitt, 1971) while the recovery rate for C. albicans in hospital patients is significantly higher. Few studies show an isolation rate of less than 30% in hospitalized patients with the mean being around 42% (Odds, 1979). Recovery of C. albicans from the intestinal tract also shows differences between normal subjects and hospitalized patients, although not as marked as the recovery rates from the oral cavity. Weighted mean recoveries of C. albicans were 14.6% and 22%, respectively, for normal subjects and patients (Mackenzie, 1962; Hughes and Kim, 1973; Odds, 1979). Recovery rates for C. albicans from the vagina show 7.8, 14.9, and 25.7% from normal subjects, patients without vaginitis and patients with vaginitis respectively (Odds, 1979). Practically every other site on the human body has been sampled for presence of yeasts, specifically C. albicans. Results of these studies show a very low (<5%) recovery rate indicating that

these organisms are probably not resident flora of these sites but merely transients (Rippon, 1974; Odds, 1979). The important point in the above studies is that many people harbor an ecosystem of commensal yeasts which have a potential to become pathogenic.

The manifestations of infections with C. albicans are as protean as those seen with syphilis. Rippon (1974) defines three major types of infections caused by C. albicans: 1) mucocutaneous disease 2) cutaneous involvement, and 3) systemic disease. Infection of the mucocutaneous membranes includes vaginitis, balinitis and alimentary tract disease usually due to improper diet (Gentles and LaTouche, 1969), poor hygiene (Rippon, 1974), antibiotic therapy (Seelig, 1966a, 1966b), or endocrine disorders (Sonck and Somersalo, 1963). These infections may clear spontaneously once the underlying factor is corrected or can be treated with topical medications such as 1% crystal violet, 0.1% hanmycin or 1% nystatin (Conant et al., 1971). Two types of patients are associated with cutaneous infection by C. albicans, those with metabolic disorders such as obesity or diabetes and those whose skin is predisposed by environmental conditions such as moisture, occlusion, and maceration. Patients with occupations such as dishwashers, fruit canners, and barmaids generally belong in this latter group (Rippon, 1974). Treatment for these afflictions is the same as that for mucocutaneous disease and elimination of the predisposing environmental factor is usually sufficient. The third major type of infection is systemic disease. This is a relatively

rare condition in an otherwise healthy individual and is usually the result of some other debilitating illness. The prognosis in this condition is poor and the death rate is high. Treatment consists of intravenous (i.v.) infusions of amphotericin B (Medoff and Dismakes, 1970), miconazole (Bannatyne and Cheung, 1978), 5-fluorocytosine (Fass and Perkins, 1971), and recently ketoconazole (Cuce et al., 1980).

Immunity to C. albicans has been the subject of a great many research reports as well as one recent review (Rogers and Balish, 1980). Since C. albicans is a microorganism of relatively low virulence (Mourad and Friedman, 1961) it becomes obvious that an intact microbial defense system, both innate as well as specific, is sufficient to control this organism. It is only when this defense system is compromised that infection and disease caused by C. albicans may occur. As has been mentioned, C. albicans is a member of the resident flora of a large portion of the population so that the potential for infection is present especially when systems that otherwise control the growth and spread of C. albicans are compromised.

When a microorganism gains entrance into the body, non-specific host defense mechanisms are the first line of defense (Smith, 1968). The blood is rapidly cleared of invading organisms by the liver, lungs, spleen, lymph nodes, and to a lesser extent, the bone marrow (Rogers, 1960). Baine et al. (1974) showed clearance of more than 90% of intravenously injected C. albicans within ten minutes in rabbits.

The vast majority of yeasts were trapped in the liver and lungs, while Sawyer et al. (1976) reported similar results in rats. They also reported that, although the yeasts were rapidly removed from the bloodstream, they were not killed. In a later paper (Sawyer et al., 1981) they showed that non-specific activation of the reticuloendothelial system by prior injection of Corynebacterium parvum, resulted in killing of the yeasts using the in vitro perfused liver model. Scanning electron microscopy showed an influx of leukocytes into the liver after C. parvum vaccination and light microscopy of the livers showed the infiltrate to be primarily mononuclear (Sawyer et al., 1981).

When invading microorganisms gain access to tissues, phagocytic cells must move to the site of challenge from the bloodstream. The polymorphonuclear neutrophil (PMN) is the first such phagocytic cell to emigrate followed by blood monocytes which differentiate into phagocytically active macrophages (Spector and Willoughby, 1963; Elsbach, 1980). Emigration of leukocytes, specifically PMN, from blood vessels to the site of infection has been the subject of many reports. It is not clear if the localization of PMN at the tissue lesion is due to chemotaxis. Harris, in 1953, showed unequivocally that colonies of living Salmonella typhi, Staphylococcus albus and Mycobacterium tuberculosis were chemotactic for granulocytes, whereas killed microorganisms were found to be non-chemotactic as were minced tissues. He concluded that emigration of white cells was a consequence of the changes in the vessel wall associated with increased vascular

permeability. Therein lies the problem, whereas chemotaxis can be unequivocally demonstrated only *in vitro*, leukocyte emigration from blood vessels can be shown only *in vivo* (Spector and Willouhby, 1963).

Chemotaxis of PMN *in vitro* was shown by Boyden in 1962. The availability of the technique he described has resulted in the characterization of several chemotactic factors as well as delineation of abnormalities of leukocyte chemotaxis in certain disease states (Snyderman *et al.*, 1975). Some of the reported chemotactic factors for PMN include zymosan (Laxalt and Kozel, 1979), complement factor C5a (Ward and Newman, 1969) and aggregated human gamma globulin (Snyderman *et al.*, 1975). Microorganisms that generate chemotactic activity include *Escherichia coli* and *Staphylococcus epidermidis* (Cutler, 1974), *Cryptococcus neoformans* (Laxalt and Kozel, 1979) and *C. albicans* (Denning and Davies, 1973; Cutler, 1977) as well as those mentioned above by Harris (1953).

Phagocytosis is facilitated by opsonins i.e. serum components such as immunoglobulins IgG and IgM (specific antibodies) and complement factor C3, since PMN and mononuclear phagocytes have receptors on their cell membrane for the Fc portion of IgG and for the C3b part of this complement factor (Van Furth *et al.*, 1978). The binding of antibodies to antigens is known to activate the serum complement system, one of whose functions is to facilitate phagocytosis (Roitt, 1974). Morelli and Rosenburg (1971) showed that complement-deficient mice did not survive as long after a lethal challenge with *C. albicans* as

mice with an intact complement system. In addition, Ferrante and Thong (1979) showed that following the removal of heat-labile opsonins by heating pooled human serum at 56 C for 30 minutes, approximately 50% loss in phagocytic activity by PMN occurred, while Ehlenberger and Nussenzweig (1977) found that C3 and IgG have synergistic roles in phagocytosis.

The action of PMN and macrophages on C. albicans has been extensively studied in vitro. Louria and Brayton (1964) showed that mouse PMN in vitro ingest virtually all C. albicans blastospores to which they are exposed within 30 minutes. This same effect was also seen with macrophages (Ozato and Uesaka, 1974). In both these studies, though, some of the yeasts grew out of the phagocytes after engulfment and thus escape intracellular destruction.

Among pathogenic species of Candida, C. albicans is found to possess the greatest ability to resist intracellular killing by outgrowth from PMN and macrophages, and C. guilliermondii is the least able (Louria and Brayton, 1964). The abilities of various Candida spp. to escape host phagocyte defenses therefore correlate with their relative virulence for laboratory animals, and with their ability to produce a filamentous growth form (Odds, 1979).

Studies which have attempted quantitative determinations of the candidacidal activities of phagocytes have shown considerable variability, with the average killing rate being about 50% of injested yeasts (Rogers and Balish, 1980).

The biochemical mechanisms of killing by phagocytes have been investigated, and several substances have been found which are candidacidal. Lehrer and Cline (1969) found that the myeloperoxidase-peroxide-halide ion system is candidacidal *in vitro* and that this system is the major component for intracellular killing of *C. albicans*. Lehrer (1972) later reported a second candidacidal mechanism in human PMN independent of the myeloperoxidase system. Elin *et al.* (1974) provided further evidence that PMN are important for defense against candidosis. Using Chediak-Higashi mice which have neutrophils with impaired capacity to kill *C. albicans*, they found an increased susceptibility to infection by *C. albicans* when compared to normal mice.

Many reports to date have dealt with acquired immunity and subsequent resistance to challenge with *C. albicans*. Injections of large amounts of high-titered anti-*Candida* antiserum have been shown to be somewhat protective in mice prior to challenge with usually lethal amounts of *C. albicans* (Mourad and Friedman, 1968) and effective in the clearing of *C. albicans* infections in humans (Hiatt and Martin, 1946). Numerous investigators have immunized experimental animals with doses of live or killed *C. albicans* yeast cells or with other microbial antigens, and then noted whether or not such immunizations protected the animals against subsequent challenge with lethal doses of yeasts. The effects of such immunizations usually appeared as a delay in mortality, rather than a prevention of mortality (Odds, 1979). Collins (1974) has suggested that the best way to study the immune

response in a vaccinated animal is to compare the rate of growth or inactivation of a sublethal challenge population of microbes in test and control groups throughout the entire infectious period.

The histopathological responses noted in experimental animal infections has been reviewed by Rogers and Balish (1980). In most cases of human candidosis, the inflammatory exudate seen in cutaneous disease is mixed, i.e., it is composed of intense infiltrates of PMN, histiocytes, and some lymphocytes (Rippon, 1974). In cases of human disseminated disease, microabcesses containing both intact and degenerating neutrophils surrounded by a zone of histiocytes are typical (Rippon, 1974). In experimental animal infections the inflammatory reaction is similar to that in humans. Pearsall and Lagunoff (1974) have described a thigh muscle model for candidosis in mice. The histopathological responses seen in this model showed an initial PMN response followed by a modest infiltration by macrophages, eosinophiles and lymphocytes. Neutrophils remained the predominant cell type and a predominance of mononuclear cells was not observed until very late in the infection, when few yeast cells remained visually (Pearsall, and Lagunoff, 1974). Rogers and Balish (1977, 1978) reported that in their kidney model for infection, the initial cellular response was neutrophilic. When the numbers of viable Candida cells are beginning to wane, the cellular infiltrate is still almost exclusively polymorphonuclear.

Engulfment and destruction by tissue macrophages or circulating PMN and monocytes appears to be the ultimate fate of most microorganisms

that penetrate as far as the tissues of the host with intact cellular defenses. Phagocytosis is a non-specific process, but its effects may be controlled and enhanced by host immune responses. As best written by Rogers and Balish (1980), "it is important that we obtain more basic information concerning C. albicans-host interactions and elucidate those innate or acquired host defense mechanisms which control the diseases caused by this unique opportunistic yeast".

Mouse footpad model

The mouse footpad model for the detection of delayed type hypersensitivity (DTH) was first described by Gray and Jennings in 1955. As originally devised, the procedure consists of inserting a short-beveled 26-guage needle into a metatarsel pad of the tensed plantar surface of a hindfoot and injecting enough antigen solution to swell an area of the foot 3 mm in diameter. In more recent applications, 27 or 30 guage needles are used and a measured volume (e.g. 0.04 ml) of antigen solution is injected (Kong et al., 1966). This technique enjoys wide adoption because it is technically easier (Crowle, 1975). Additionally, the footpad test may be more sensitive than the classic skin flank test because the antigen is held in situ longer (Crowle, 1959). This was confirmed by Oghiso and Matsuoka (1979) who followed distribution of colloidal carbon in mice injected by various routes. A typical footpad DTH reaction will become visible due to induration at about 6 h after challenge, peak at 18 to 24 h,

still persist strongly at 48 h, and then recede, to disappear after another 1 to 2 days (Collins and Mackaness, 1968). Most frequently, this delayed reaction will be preceded by a soft edematous humoral antibody swelling, first appearing at about 2 h, peaking at 3 to 4 h, and usually disappearing by 6 h (Collins and Mackaness, 1968).

Footpad reactions can be usually read subjectively by comparing the test foot with the opposite foot, which either is untreated or has been injected with antigen solvent only (Gray and Jennings, 1955). Alternatively, the response can be measured objectively with calipers (Youdim et al., 1973), a skin thickness dial guage (Rifkind et al., 1976) or, for the finest distinction, by the amount of a fluid that the swollen foot will displace in a plethysmograph (Pearson et al., 1971). These objective measurements can be made by comparing swelling in the experimental foot with that of the control foot or by subtracting the control value (measurement taken before injection) from the experimental readings (Rifkind et al., 1976). The nature and quantity of the antigen are important. Tests with undiluted old tuberculin (OT) are unsatisfactory because the glycerin in OT causes severe nonspecific swelling (Gray and Jennings, 1955) while Flynt et al. (1975), showed detectable responses to 1:10,000 dilutions of merthiolate which is used as a preservative in coccidioidin and other antigens. Because some antigens are toxic (Anacker et al., 1969), the lowest test doses are used that will reliably detect DTH. Effective quantities must be determined empirically for the antigen used. For most antigens employed,

such test doses are only a few micrograms (Anacker et al., 1969; Collins and Mackaness, 1968) or a few organisms (Patel and Lefford, 1978).

The histological features of DTH is a predominantly mononuclear cell infiltrate (Gell and Hinde, 1954) whereas a reaction in which polymorphonuclear cells predominate is widely interpreted as an Arthus reaction (Crowle, 1975). There are important exceptions. In DTH reactions to polysaccharides in mice, monocyte infiltration is heavy, but the polymorphonuclear cell maintains numerical superiority from early in its reaction throught its peak (Kong et al., 1966). This is not due to an Arthus reaction, for it is passively transferred with lymphoid cells but not antiserum. Rifkind et al. (1976) reported that in mice sensitized with Coccidioides immitis mycelial antigen, there was primarily a neutrophilic leukocyte response at 24 and 48 h along with a significant mononuclear component in the infiltrate which was not seen in non-sensitized mice. The cellular infiltrate in mice actively sensitized with antigen was indistinguishable from that seen in mice passively sensitized with immune spleen cells. Essentially, no mononuclear cells were observed in footpads of mice given only immune serum (Rifkind et al., 1976).

The DTH reaction observed in mice is antigen specific. Mice immunized with C. immitis antigen reacted when footpad challenged with homologous antigen but failed to react with C. albicans antigen (Rifkind et al., 1976). Although there may be cross-reactivity between

similar antigens (e.g., Salmonella enteritidis and S. gallinarum) most DTH reactions are appropriately specific (Crowle, 1975). For example, mouse DTH can demonstrate the differences between bacterial species of the same genus (Collins and Mackaness, 1968), discriminate mumps from influenza viruses (Feinstone et al., 1969) and help to identify haptenic molecules (Bekierhunst and Yarkoni, 1973).

There have been other models devised for detection of DTH in the mouse, some before the footpad technique and some after. The first satisfactory evidence that mice could develop DTH was by Hart et al. in 1952. They showed that mice chronically infected with virulent tubercle bacilli could be fatally shocked with purified protein derivative (PPD).

Later, Kircheimer and Malkiel (1953), demonstrated that mice could be sensitized to tuberculin without virulent infection. This reactivity was elicited by skin testing the mice, inherently difficult due to the thinness of the mouse skin. Although this skin test technique is readily mastered, it is not as widely used as footpad tests despite the advantages of offering more test sites on an animal, allowing easy quantitative objective measurement, and being simpler and quicker to perform (Crowle, 1959).

A more recent model for DTH detection, the mouse-thigh lesion has been developed by Pearsall and Lagunoff (1974) and Pearsall et al. (1978). With this technique, a large number of viable C. albicans is injected intramuscularly into the thigh. The hypersensitive response

is measured as the subsequent swelling of the thigh. The thigh lesions can also be followed histologically. A criticism of this model can be made because a very large number of viable C. albicans yeast cells (5×10^8) must be used. Leunk and Moon (1979) reported toxin-like properties of C. albicans when administered i.v. to mice in high doses (4.5×10^6). These effects were not seen with the same dosage of heat-killed cells.

Attempts to follow the numbers of intralesional organisms have, until recently, been mostly qualitative observations, although a common procedure used in assessing systemic spread of organisms after i.v. challenge is to homogenize selected organs and determine viable cell counts. Patel and Lefford (1978) noted the presence or absence of acid-fast bacilli in tissues of Mycobacterium leprae sensitized mice after footpad challenge while Giger et al. (1978) reported relative numbers of fungal elements in cutaneous lesions of mice sensitized with live C. albicans. Louria et al. (1963) reported, that in i.v. injected animals, organisms became harder to detect microscopically as the inflammatory reaction intensified.

In 1974, Lepper studied qualitatively as well as quantitatively the cellular components of the inflammatory response in the skin during the course of a primary experimental dermatophyte infection but only noted the presence or absence of fungal structures in relation to the types of leukocyte infiltrate. Green and Balish (1979) took this experiment further by quantitatively following fungal proliferation

and elimination during lesion development by homogenizing and culturing skin infected with Trichophyton mentagrophytes. They showed that lesion severity, in terms of gross tissue damage, occurred at the same time as peak fungal load and that reinfection with the same organism resulted in accelerated lesion development, probably as a result of an anamnestic hypersensitivity reaction, but with no corresponding peak fungal load. The infecting organism was eliminated at the same rate in both primary and secondary infections. They suggested that skin homogenization may be a more sensitive method of determining fungal persistence because: 1) organisms may be difficult to see microscopically, and 2) the inability of differentiating viable from non-viable organisms (Green and Balish, 1979).

The first report using mouse footpad injections to produce a self-limiting localized infection was that of Shepard in 1960. The technique has been of inestimable value in the development of new chemotherapeutic agents and in assessing the rate at which Mycobacterium leprae are killed during treatment of patients (Shepard, 1971 and Shepard et al., 1968). The in vivo footpad microbicidal technique has also been used by Patel and Lefford (1978) as evidence for cell-mediated immunity (CMI) to M. leprae antigens in sensitized mice. After sensitization, mice were footpad challenged with viable Listeria monocytogenes. Twenty-four hours later, the mice were sacrificed, the injected foot was homogenized and appropriate dilutions were plated. Reduction in viable cells per foot was evidence for CMI since one of

the hallmarks of CMI is nonspecific resistance to listeria (Mackaness et al., 1974). Collins et al., (1978) also used the mouse footpad culture method to study immunity to mycobacterial infection. They injected footpads of mice with a readily cultivable Mycobacterium strain, M. marinam. The onset of cell-mediated immunity was well correlated with a concurrent decrease in viable cells cultured from the homogenized foot. The above experiments show that the in vivo mouse footpad assay model lends itself to further study with different microorganisms which can lead to a better understanding of host-parasite interactions.

Cyclophosphamide

Efforts to modify the chemical structure of the nitrogen mustards to achieve greater selectivity for neoplastic tissues led to the development of cyclophosphamide (Arnold et al., 1958). The first use of this family of compounds was in gas warfare during World War I. Modern interest in these compounds as anticancer agents is a byproduct of studies on war gases during World War II. Gilman and his colleagues, after noting the toxicity of simple alkylating agents to lymphoid tissues and rapidly dividing cells, suggested that alkylating agents might have a therapeutic effect against tumors of lymphoid origin (Gilman and Philips, 1946). The suggestion led to studies on neoplasms in experimental animals and subsequently to clinical tests, which demonstrated that one of the mustards had activity against Hodgkin's

disease. This agent was the first synthetic compound clearly shown to possess antitumor activity in humans. These results led to the synthesis of hundreds of other compounds possessing alkylating activity. It was not until 1965 that Schmidt and colleagues critically evaluated 94 alkylating agents against 25 different rat and mouse neoplasms (Schmidt et al., 1965). The outstanding representatives in nearly every test system were the isomeric phenylalanine mustards and cyclophosphamide (2-[bis(2-chloroethyl)amino] tetrahydro-2H-1,3,2-oxazaphosphorine 2-oxide). Among the trade names are the following: Cytoxan (United States), Exdoxana (England) and Procytox (Canada).

Cyclophosphamide is prepared by treating N, H-bis (2-chloroethyl) phosphoramidic dichloride with an equimolar amount of 3-amino-1-propanol in the presence of two molar equivilents of triethylamine (Arnold et al., 1958). It is soluble in water, ethanol, benzene, acetone, and ether (Arnold et al., 1958). As determined by an assay with leukemic mice, cyclophosphamide is stable in solution for at least 4 weeks (Vietti et al., 1973).

The initial success of cyclophosphamide as an antitumor agent in experimental tumor systems (Arnold et al., 1958) and the demonstration that it was ineffective against cells in vitro (Foley et al., 1960) led to numerous attempts to explain the enzymatic mechanism by which activation occurs. The most favored mechanism for activation of cyclophosphamide was based on the elevated phosphoramidase activity in certain tumor tissues which would cleave the compound and release

a biologically active (cytostatic) product (Hill, 1975). Foley et al. (1960, 1961) tested blood serum and homogenates of various normal and neoplastic tissues of rats previously injected with cyclophosphamide and noted a product that was toxic to mammalian cells in culture, but found only in the liver and the blood. Incubation of cyclophosphamide with homogenates showed only the liver produced an inhibitory substance, indicating that this organ was the primary site of activation for the agent. Perfusion of an isolated rat liver with whole rat blood containing cyclophosphamide gave a high concentration of toxic material. Liver slices produced active metabolites only when incubated in the presence of oxygen while slices of other tissues were essentially ineffective (Brock and Hohorst, 1967).

Chromatography of serum from rats injected with labeled cyclophosphamide revealed two metabolites, which could also be found after incubation of the drug with slices of rat liver (Hill, 1975). Further analysis showed that the major metabolite contained phosphorus. While the structure of the activated form of cyclophosphamide is still unsettled, studies using isophosphamide have shown that this compound is metabolized by mouse liver microsomes in a manner similar to that for cyclophosphamide (Hill et al., 1973). The activated product of isophosphamide oxidation binds to deoxyribonucleic acid and inhibits thymidine incorporation into deoxyribonucleic acid by leukemic L1210 cells (Allen and Creaven, 1972).

After injection into mice cyclophosphamide is rapidly metabolized with the rate based on the size of the dose applied versus the

cytostatic activity demonstrable in serum (Hill, 1975). The half-life of cyclophosphamide in mouse serum is about 20 min and only 5% of a 100 mg/kg dose is excreted unchanged in a 24 h period (Hill, 1975). Cyclophosphamide is found in the brain of mice as soon as five min after an i.p. injection (Mellet, 1966). The whole-body autoradiographic technique revealed that cyclophosphamide accumulated within 30 min of an i.p. injection in the liver, gallbladder, small intestine and kidneys of mice with a later accumulation in the thymus, spleen and testes (Hill, 1975).

The presence of a foreign compound in the tissues of an animal undoubtedly causes many changes in metabolism. During the past 20 years, the influence of cyclophosphamide on tissues and organ systems has been extensively studied. Tumors of mice treated with cyclophosphamide have a lower rate of *in vitro* glycolysis 4 h after treatment (Wright *et al.*, 1960) but after 72 h glycolysis is no longer operative. The conclusion was that tumor inhibition by cyclophosphamide is a process independent of glycolytic activity. Preliminary experiments in the field of lipid metabolism have given no clues to the mechanism of action of cyclophosphamide. The effect of this agent on lipid content of tumors is dependent on the type of tumor studied (Hill, 1975). Cyclophosphamide has demonstrable effects on the cellular processes involved in the production of ATP. Some of these have been implicated as fundamental to the action of the agent. The nicotinamide adenine dinucleotide (NAD) levels of tissues of animals treated with

cyclophosphamide have been the subject of several investigations. In mice, high does of cyclophosphamide decrease the NAD glycohydrolase activity of the spleen (Tsukagoshi et al., 1968). Liss and Palme (1965) reported a decrease in NAD levels in Ehrlich ascites cells, but this fall in NAD content occurred only after a change in the deoxyribonucleic acid/ribonucleic acid ratio and is thus probably not the primary mechanism for toxicity.

There seems to be common agreement that cyclophosphamide inhibits the incorporation of precursors into DNA; this has been considered as a possible primary effect of the drug. Liss and Palme (1963) found that incorporation of H^3-thymidine into the deoxyribonucleic acid (DNA) of Ehrlich ascites cells and spleen of mice is reduced 50% after treatment with a moderate dose of cyclophosphamide, while Short et al. (1972) showed decreased DNA synthesis by mouse embryos at all times after 6 h post-injection. The effects of cyclophosphamide on chromosomes are probably related to its action on cellular DNA. Chromosomal disturbances have been observed in ascites tumor cells and mouse bone marrow (Arrighi et al., 1962). Leukocyte cultures from patients treated with cyclophosphamide show chromosomal abnormalities (Arrighi et al., 1962). In vitro treatment of human leukocytes does not result in chromosomal damage (Hampel et al., 1966) while another publication states that chromosomal and polyploidy changes are evident in such cells after exposure to cyclophosphamide (Nasjleti and Spencer, 1967). The results of this last study imply

that cells in culture are capable of converting cyclophosphamide to an active form, a fact which has not been conclusively demonstrated (Hill, 1975). The list of effects continues to grow, but as yet the primary action responsible for cytotoxicity of cyclophosphamide is not known with certainty.

In all animals tested, cyclophosphamide produces severe and potentially lethal depression of hematopoietic stem cells. Also, affected are cells of the lymph nodes (Turk and Poulter, 1972), spleen (Turk and Poulter, 1972), thymus (Castaldi et al., 1972) and bone marrow (DeWys et al., 1970). The total marrow cell count and the peripheral blood leukocyte count of mice fall progressively for three days after cyclophosphamide administration but both return to normal after a few more days (DeWys et al., 1970). The number of erythrocytes in mice is not greatly changed by cylophosphamide treatment (Valeriote et al., 1968) but rapidly proliferating erythroid cells are more sensitive (Blackett and Adams, 1972). Peripheral granulocytopenia is seen in rats and mice following injection of cyclophosphamide (Shadduck and Nunna, 1971) but granulocytosis is seen during hematologic recovery (Host, 1966). Neutropenia is also seen in cyclophosphamide treated dogs and monkeys (Schmidt et al., 1965). The hematological effects of cyclophosphamide on humans are similar to those seen with experimental animals (Pegg, 1963).

Cyclophosphamide has been used extensively as an immunosuppressant of both the humoral and cellular immune systems in experimental

animals. Early experiments showed decreased antibody production to human erythrocytes in cyclophosphamide pretreated mice (Frisch and Davies, 1966). Small doses are most immunosuppressive when given on the same day while large doses are effective even after three days subsequent to antigen injection (Frisch and Davies, 1966). In a now classical experiment they showed that a large, single dose of cyclophosphamide given to mice can result in complete suppression of the immune response to a previously administered erythrocyte antigen but not to a different one given 1 to 2 days later. Aisenberg and Wilkes (1967) using the Jerne plaque assay showed that cyclophosphamide alone kills the hemolysin-producing cells (B lympocytes), while non-proliferating cells are killed at a much lower rate. Total recovery of immune competence may require more than 30 days (Marbrook and Baguley, 1971). Mice treated with cyclophosphamide will respond normally to sheep erythrocytes if immunocompetent cells from other mice are transferred at the same time (Santos, 1966).

The effects of cyclophosphamide on the cellular immune system are similar to those of the humoral immune system. The drug prolongs the survival of both skin allografts and homografts on mice, especially if it is given to the animals between one day before and two days after transplantation (Fox, 1964; Floersheim, 1969). Graft vs. host disease induced in mice by injection of spleen cells from another strain is not as lethal in animals treated with cyclophosphamide (Levy, 1973). Sharma and Lee (1977) showed functional T-cell

deficiency accompanied by a marked degeneration in the thymus dependent areas of the spleen as well as the thymus itself in chickens injected with cyclophosphamide.

Most recent reports refer to the exclusivity of cyclophosphamide as a B-cell suppressant but since cooperation between B and T cells is required in the immunological response, the destruction of either could lead to tolerance.

The effect of cyclophosphamide pretreatment on infections is a general enhancement of the infectious process. Numerous reports in the literature show, in general, a decreased resistance to microbial challenge. This effect is seen with microorganisms such as Staphylococcus aureus (Sharbaugh and Grogan, 1969) influenza virus (Hurd and Heath, 1975) Mycoplasma pulmonis (Singer et al., 1972) Nocardia asteroides (Beamon and Maslan, 1977) and Candida albicans (Moser and Domer, 1980).

MATERIALS AND METHODS

Animals

Female HA(ICR) mice weighing 28 to 32 g were purchased from Harlan Laboratories, Indianapolis, Ind. Animals were maintained under standard laboratory conditions with Wayne Lab-Blox (Allied Mills, Inc., Chicago, Ill.) and water available *ad libitum*.

Microorganisms

The strain of *Candida albicans* used in this study was a fecal isolate obtained from the Olin Health Center, Michigan State University. Identity was confirmed by typical assimilation patterns using the method of Land *et al*. (1975). Adjunct tests, such as germ tube formation and chlamydospore production, were also performed. Stock cultures were maintained on Sabouraud dextrose agar (SDA) slants at 25 C. When live cells were used, the inoculum was prepared by transfer of a loopful of the stock to 100 ml of trypticase soy broth (Difco) supplemented with 4% d-glucose and incubated for 16-20 h at 37 C on a rotary shaker. Yeast-phase cells were harvested and washed three times in sterile saline (0.85% NaCl). Haemocytometer counts were used to standardize the inoculum and pour plates of 10-fold dilutions in SDA confirmed any adjustments made.

Killed cells were prepared by heating the yeast suspension in a 60 C water bath for 4-5 h. Non-viability was ensured by plating an aliquot onto SDA and incubation at 35 C for three days. The

suspensions of heat-killed cells were frozen at -20 C and thawed immediately before use.

Another species, C. krusei was obtained from the stock collection of Dr. E.S. Beneke of Michigan State University. This species was used for comparative studies with C. albicans. Preparation of inoculum was the same as for C. albicans.

Sensitization

Four weekly doses of 1 x 10^6 heat-killed C. albicans in 0.1 ml sterile saline were injected subcutaneously at alternating ventral areas of mice. Sensitivity was assayed 1 week after the final injection using random animals picked from the group.

LD_{50} procedure

The computation of the LD_{50} was according to the procedure of Reed and Muench (1938). Mice were injected (six per group) via a lateral tail vein with a 0.1 ml suspension of 10^3, 10^4, 10^5, or 10^6, washed C. albicans yeast-phase cells per ml using a 1 ml plastic syringe with a 27 guage needle. The animals were monitored daily and the experiment was terminated after 30 days. The accumulated total of deaths per groups during this period was used as the basis for computing the median lethal dose.

Prepartion of bovine anti-Candida albicans antiserum

A yearling heifer (MSU #228) was vaccinated by the following procedure. Two weekly subcutaneous injections of 2.5 x 10^9 heat-killed

C. albicans suspended in sterile saline were administered at different shaved sites. Two weeks after the second injection, the cow was skin tested intradermally with 1 ml of a 1:5 saline dilution of the same antigen. Increases in skin thickness and areas of inflammation were measured with calipers at 0 h (control) and at 24, 48, and 72 h after antigen injection. Results showed a definite increase in skin thickness surrounded by an area of inflammation at all times subsequent to injection. A control site injected with sterile saline showed no measurable reaction. Serum collected at the time of skin testing showed a tube agglutination titer of 2560. Fetal bovine serum (Grand Island Biological Co., Grand Island, N.Y.) was used as the negative bovine control in both the immunodiffusion and agglutination procedures.

Footpad sensitivity assay

The sensitivity of mice to C. albicans was assayed by means of the footpad swelling test (Rifkind et al., 1976). In footpad tests, 2×10^5 heat-killed C. albicans suspended in 0.04 ml sterile saline was injected intradermally into the right hind footpad. The left hind footpad, injected with an equal amount of sterile saline, served as the control. The thickness of both feet were measured with a micrometric caliper (Scientific Products, Rochester, Mich.). Measurements were made prior to and immediately after (0 h), followed by 4, 24, 48, and 72 h after injection. The net increase in footpad

response was determined by subtracting the increase in the control foot from that of the experimental foot.

Serological tests

Sera from mice before and after sensitization were tested for the presence of precipitating and agglutinating anti-candidial antibodies. The method of Ouchterlony (1949) was used in the immunodiffusion assay for precipitating antibodies. The diffusion medium consisted of 1.0% agarose, 7.5% glycine and 0.85% NaCl. The medium was autoclaved, cooled to 50 C and 2.5 ml pipetted onto clean, level microscope slides (3 x 1 inch). After the agar had solidified, wells were cut using a gel diffusion punch (Diagnostic Products, Inc., Cincinnati, Ohio). The agar plugs were removed from the wells using a pasteur pipette and mild suction. Antigen (Oidiomycin; Hollister-Stier, Inc., Spokane, Wash.) was added to the outer wells. Serum to be tested was placed in the center well. Positive control serum from the hyperimmune cow was included with each run. The slides were incubated at 37 C in a moist chamber for 24 h and examined for precipitin lines.

Agglutinin titers with homologous and heterologous antigens were determined by the tube agglutination test (Sweet and Kaufmann, 1970) in parallel with the immunodiffusion procedure. Antigens were adjusted to 5×10^7 heat-killed cells per ml with a haemocytometer. Two-fold dilutions of the test serum in 0.5 ml volumes were mixed with an equal volume of the standardized cell suspension. The tubes were incubated

in a 37 C waterbath for 48 h after which they were read for visible agglutination.

Cyclophosphamide pretreatment

Mice were injected intraperitoneally (i.p.) with appropriate doses of cyclophosphamide (Cytoxan; Mead Johnson Laboratories, Evansville, Ind.) dissolved in saline. In all cases, the mice were used in experiments at least 3 days and no longer than 5 days after injection.

Leukocyte determination

Blood samples were obtained from a lateral tail vein. White cell counts were performed using Unopettes (#5853; Becton Dickinson and Co., Rutherford, N.J.). Cells were counted in an improved Neubauer chamber (haemocytometer). Differential leukocyte counts were performed on air dried smears stained by the Wright method. Cell types were expressed as the percentage of 100 total cells counted multiplied by the total white cell count.

Footpad assay with live Candida albicans

Mice were challenged with a live dose of C. albicans injected into the right hind footpad. At appropriate time intervals, groups of mice were killed by cervical dislocation. The thickness of the foot was measured (see above) and foot removed with sterile scissors. The foot was surfaced sterilized by dipping in 70% ethyl alcohol and

placed in a 100 ml graduate cylinder. The volume was adjusted to 100 ml with sterile saline and the foot was homogenized in a Virtis "45" Hi-Speed Homogenizer (The Virtis Co., Gardiner, N.Y.) for 2 minutes at medium speed. Quantitative SDA pour plates were made, in duplicate, using 10-fold serial dilutions of the homogenate in saline. The plates were incubated at 37 C for 48 h and the number of colony forming units (CFU) were counted manually on a Quebec Colony Counter (American Optical, Buffalo, N.Y.). The surviving yeasts were expressed as the Log_{10} present in the homogenate.

Control experiments were performed to ascertain dissemination of the yeast inoculum to other organs. Samples of liver, lung, spleen and kidneys were homogenized with a glass-teflon homogenizer in 10 ml sterile saline, and counted as above.

Histopathology

The foot that had been inoculated with antigen was removed. The plantar surface (footpad) was carefully excised and fixed in buffered 10% formalin. The tissues were embedded in paraffin, sectioned at 6 microns, and stained with both hematoxylin-eosin (H & E) and periodic acid-Schiff (PAS) stains.

Isolation of mouse polymorphonuclear neutrophils

The procedure for preparation of mouse polymorphonuclear neutrophils (PMN) used in these experiments was similar to that of Cutler (1974) with minor modifications.

Mice were injected i.p. with 3 ml of 0.5% glycogen (Sigma Chemical Co., St. Louis, Missouri) in 0.85% NaCl. After 3 to 4 h, 3 ml of tissue culture medium 199 (M199; Grand Island Biological Co., Grand Island, N.Y.) plus a 5 USP units/ml sodium heparin (United States Biochemical Corp., Cleveland, Ohio) was injected i.p. After 5 to 10 minutes, the mice were killed and the abdominal skin was cut away, being careful not to cut the peritoneal membrane. The peritoneal fluid, which consisted of tissue culture medium with peritoneal exudate cells, was aspirated into siliconized test tubes using a 3 ml syringe with an 18 guage needle. Siliconized tubes were used throughout these experiments to prevent adherance of leukocytes to the test tube walls. The exudate cells were pelleted by centrifugation at about 200 x g for 10 min., washed once in fresh M199 and resuspended in M199 + 5 units/ml heparin + 10% fetal calf serum. Viability was determined by trypan blue dye exclusion and the percent PMN in the peritoneal cell suspension was determined by differential counts of Wright stained smears. Cells were counted using a haemocytometer. The suspension was kept on ice until ready for use in either the phagocytosis or candidacidal assay.

Phagocytosis by peritoneal cells

Neutrophils were allowed to adhere to glass by the following procedure. One ml aliquots of the cell suspensions containing approximately 1 x 10^6 PMN were placed onto sterile 22 x 22 mm glass cover slips. The cover slips were incubated for 1 h at 37 C after which nonadherent cells were removed by gentle, repeated rinses of

fresh M199. One ml portions of M199 containing 1-2 x 10^5 viable yeast cells were placed onto the cover slips and incubated at 37 C for 1 h. After incubation, the cover slips were fixed in 100% methanol for 10 min, stained by the Wright method, and attached to microscope slides with Permount (Fischer Scientific Co., Fair Lawn, N.J.). The cover slips were examined under oil immersion for ingested yeast cells. For estimating germ tube production, 100 phagocytosed yeast cells were counted and germ tube producing cells were expressed as a percentage. For a control, 100 extracellular yeast cells were examined for germ tube production.

An additional experiment was designed to determine if incubation of PMN from normal mice in various concentrations of cyclophosphamide would alter their germ tube inhibition ability. In this experiment, the PMN were harvested as before but were incubated for 1 hr in M199 plus 0.01, 0.1 and 1 mg/ml cyclophosphamide while allowed to adhere to glass cover slips. The yeast cells were added after this time and the cover slips were incubated for an additional hour. The cover slips were then fixed, stained and counted as above.

PMN candidacidal assay

Neutrophils were assessed for killings of yeast cells in the following manner. To a 0.9 ml suspension containing 1 x 10^6 mouse peritoneal PMN was added 1 x 10^5/0.1 ml viable C. albicans cells. The leukocyte and yeast cell suspension was incubated at 37 C on a rotary

shaker for 2 h. A control tube contained medium with all components except leukocytes.

Following incubation, the suspension was diluted in 9 ml saline and homogenized in a sterile glass-teflon homogenizer. Quantitative SDA pour plates were made using 10-fold serial dilutions of the control and experimental suspensions in saline. The plates were incubated for 48 h at 37 C and counted manually. The percentage of viable yeast cells was expressed according to the following formula:

$$N = \frac{\text{number of CFU in experimental tube}}{\text{number of CFU in control tubes}} \times 100$$

Statistics

Results were analyzed using the Students t test. All data was reported with its standard deviation.

RESULTS

Immune responses in sensitized mice

Groups of mice were actively sensitized with C. albicans or C. krusei heat-killed yeast cells. Random animals from each sensitized group were selected and assayed 1 week after the final subcutaneous injection of antigen. Serum was collected from some of the animals for serological studies while other mice were tested for footpad reactivity.

Sera collected from mice before and after sensitization were tested for precipitating and agglutinating antibodies. Precipitin reactions with oidiomycin were strongly positive using sera from sensitized mice while non-sensitized mouse sera showed no reaction. The specific bovine antiserum, included as a positive control, consistently show precipitins. Sera from mice sensitized with C. krusei yeast cells showed no precipitins with the C. albicans antigen. Agglutinating antibodies were not detected in any of the mouse sera using the tube agglutination technique with heat-killed C. albicans yeast cells. A strong agglutination titer (2560) was seen with the bovine antiserum. In addition, a weak agglutination titer (20) was seen using heat-killed C. krusei yeast cells and the bovine antiserum indicating little antigenic cross-reactivity between C. albicans and C. krusei.

Reactivity of mice to the sensitizing antigen was also assayed utilizing the footpad swelling technique. Preliminary experiments

showed that the minimum amount of yeast antigen needed to elicit a response represented ca. 2 x 10^5 yeasts per 0.04 ml (data not shown). This concentration of antigen was used in all subsequent experiments. The data in Table 1 show the net footpad increase at all times except 0 h is significantly greater in mice sensitized with antigen ($p < 0.001$) than in those with no prior sensitization. At 4 h, footpad reactivity in non-sensitized mice was approximately one-half that seen in the sensitized group. Between 4 and 24 h, the footpad reaction was still increasing in the sensitized group while the reactions in the non-sensitized mice waned. By 48 h, the footpad measurements in the non-sensitized mice had returned to normal. The 48 h measurement in the sensitized group showed the footpad still very swollen but by 72 h, the footpad had essentially returned to normal size. To show that this reaction was specific for the sensitizing antigen, groups of mice were sensitized with another species of Candida, C. krusei.

Table 2 shows the specificity of footpad reactions in mice challenged with homologous antigen and no reactivity in those animals challenged with heterologous antigen ($p < 0.001$).

Overall, these data indicate that mice sensitized with heat-killed yeast cells: 1) produce detectable antibody, and 2) show specific footpad reactivity when challenged with homologous antigens. In addition, this specific footpad responsiveness remained for at least 18 weeks after the final sensitization dose although serum antibody became undetectable by that time (data not shown).

Table 1. Footpad reactivity in non-sensitized and sensitized mice after injection with 2×10^5 heat-killed C. albicans.

Sensitizing antigen	Net footpad response in recipients[a]				
	0 h	4 h	24 h	48 h	72 h
C. albicans[b]	0 ± 0	61 ± 14	105 ± 17	61 ± 20	17 ± 4
None	0 ± 0	33 ± 10	16 ± 8	5 ± 6	1 ± 5

[a]mean increase in footpad thickness ± standard deviation in 0.01 mm; six animals per group.

[b]mice were sensitized with 4 weekly subcutaneous injections of 10^6 heat-killed C. albicans yeast cells.

Table 2. Specificity of footpad response in non-sensitized and sensitized mice after injection with 2×10^5 heat-killed yeast cells.

Sensitizing[b] antigen	Net footpad response in recipients[a]							
	4 h		24 h		48 h		72 h	
	C. albicans	C. krusei	C. albicans	C. krusei	C. albicans	C. krusei	C. albicans	C. krusei
C. albicans	61 ± 14	22 ± 8	105 ± 17	13 ± 7	61 ± 20	9 ± 7	17 ± 4	5 ± 5
C. krusei	29 ± 6	58 ± 5	15 ± 3	100 ± 17	9 ± 6	42 ± 11	4 ± 3	15 ± 3
None	33 ± 10	32 ± 8	16 ± 8	11 ± 5	5 ± 6	5 ± 5	1 ± 5	2 ± 4

[a]mean increase in footpad thickness ± standard deviation in 0.01 mm; six animals per group.

[b]mice were sensitized with 4 weekly subcutaneous injections of 10^6 heat-killed yeast cells.

Survival of mice after intravenous injection with viable C. albicans

The LD_{50} values for the non-sensitized group is approximately the same as that of the sensitized group (Table 3). Pretreatment with 200 mg/kg cyclophosphamide significantly decreased the animals' ability to survive the challenge (> 100 fold). Mice pretreated with 100 mg/kg of cyclophosphamide showed a greater ability to survive when compared to the 200 mg/kg group although the median lethal dose value was much lower than observed in those groups not receiving cyclophosphamide.

White blood cell kinetics in non-sensitized, sensitized and cyclosphosphamide pretreated mice

The total white blood cell count (WBC) for non-sensitized mice was approximately 8289 cells/mm^3 of which 17% (1467) were PMN, 76% (6563) were lymphocytes, and 3% (259) were monocytes (Table 4). The WBC and differential for sensitized mice were essentially the same as that for non-sensitized mice. Seventy-two hours after injection with 100 mg/kg cyclophosphamide the WBC decreased to 5491 cells/mm^3 with the greatest decrease seen in the calculated number of PMN. The total numbers of lymphocytes and monocytes, although depressed when compared to non-drug treated animals was not statistically significant ($p > 0.05$). An even greater decrease in WBC was observed in mice pretreated with 200 mg/kg of cyclophosphamide. The WBC was 4255 cells/mm^3 with 6% (253) PMN, 92% (3816) lymphocytes and

Table 3. LD_{50} values in non-sensitized, sensitized and cyclophosphamide (CY) pretreated mice, after intravenous injection.

Experimental	LD_{50}[a]
Non-sensitized	3.4×10^5
Sensitized[b]	3.3×10^5
CY (100 mg/kg)[c]	8×10^3
CY (200 mg/kg)	$<1 \times 10^3$

[a]mice (6 per group) were injected with varied dilutions of live washed C. albicans cells in saline and observed for 30 days.

[b]mice were sensitized with 4 weekly injections of 10^6 heat-killed C. albicans yeast cells.

[c]mice received a single intraperitoneal injection of cyclophosphamide 3-5 days prior to injection.

Table 4. White blood cell kinetics in non-sensitized, sensitized and cyclophosphamide (CY) pretreated mice.

		Differential[a]		
Experimental	Total WBC/mm^3	PMN	Lymphocytes	Monocytes
Non-sensitized	8289 ± 1132[b]	1467 ± 115	6563 ± 1112	259 ± 32
Percent[c]		17 ± 1	76 ± 6	3 ± 1
Sensitized[d]	8897 ± 1152	1444 ± 169	7229 ± 1281	224 ± 33
Percent		16 ± 2	81 ± 7	3 ± 1
CY-100 mg/kg[e]	5491 ± 1180[ef]	358 ± 117[f]	4923 ± 1140	210 ± 20
Percent		7 ± 2	91 ± 5	2 ± 1
CY-200 mg/kg	4255 ± 1155[ef]	253 ± 21[f]	3816 ± 1204[g]	186 ± 33[g]
Percent		6 ± 1	92 ± 5	2 ± 1

[a]each value represents at least 5 experimental determinations.

[b]mean ± standard deviation.

[c]relative percent of 100 cells counted.

[d]mice were sensitized with 4 weekly subcutaneous injections of 10^6 heat-killed C. albicans yeast cells.

[e]mice received a single intraperitoneal injection of cyclophosphamide 72 h prior to bleeding.

[f]$p < 0.001$ vs. non-sensitized mice

[g]$p < 0.02$ vs. non-sensitized mice.

2% (186) monocytes. Again, the greatest reduction in cell type was seen in the PMN ($p < 0.001$) although all cell types were significantly reduced ($p < 0.02$). Eosinophils were occasionally seen but always comprised less than 1% of the total white cell count.

Footpad reactivity in non-sensitized and sensitized mice injected with live yeast cells

Mouse hind footpads were inoculated with washed viable C. albicans yeast cells. At 0, 4, 24, 48, and 72 h, groups of mice were sacrificed. The footpad thickness was measured, and the foot removed. Viable yeast cells remaining in the foot were determined by pour plates of the foot homogenate.

Table 5 shows the results of footpad measurements in non-sensitized and sensitized mice injected with live yeast cells. The non-sensitized mice exhibited a maximum response at 4 h. The footpad decreased to approximately the same thickness at both 24 and 48 h with further decrease by 72 h. At 96 h, the foot had returned to its pre-injection size (data not shown). The footpad thickness response in the sensitized mice was significantly greater ($p < 0.01$) than that of the non-sensitized group at all time points with the exceptions of the 0 h and the 72 h readings. This group displayed an equally intense response at 4 h and 24 h followed by a moderate reduction at 48 h. At 72 h, the footpad swelling was on the decline and returned to normal by 96 h.

Table 5. Footpad reactivity in non-sensitized and sensitized mice after injection with 2×10^5 viable C. albicans.

	Net footpad response in recipients[a]				
Sensitizing antigen	0 h	4 h	24 h	48 h	72 h
C. albicans	0 ± 0	129 ± 12	131 ± 10	101 ± 16	41 ± 17
None	0 ± 0	101 ± 11	49 ± 10	51 ± 10	32 ± 10

[a]mean increase in footpad thickness ± standard deviation in 0.01 mm; six animals per group.

[b]mice were sensitized with 4 weekly subcutaneous injections of 10^6 heat-killed C. albicans.

Table 6. Viable yeast cells present in foot homogenate of non-sensitized and sensitized mice after injection with 2×10^5 viable C. albicans.

	CFU/foot at hours post-injection				
Sensitizing antigen	0 h	4 h	24 h	48 h	72 h
C. albicans	5.2 ± .04	5.1 ± .14	4.2 ± .06	3.4 ± .25	3.2 ± .07
None	5.2 ± .05[a]	4.7 ± .13	4.1 ± .15	3.3 ± .09	3.1 ± .05

[a]mean $\log_{10}$ colony forming units (CFU) ± standard deviation; 5 animals per data point.

[b]mice were sensitized with 4 weekly subcutaneous injections of 10^6 heat-killed C. albicans yeast cells.

Viable cell counts in non-sensitized mouse footpads are shown in Table 6. The non-sensitized group showed a steady decrease in the number of live yeast cells present in the injected foot. By 72 h, less than 1% of the original inoculum remained. The sensitized group showed a similar decrease in viable cell counts. As with the non-sensitized group at 72 h, greater than 99% of the original inoculum was no longer viable.

Control experiments were also performed to determine if any dissemination of the injected inoculum occurred. Rarely were yeasts cultured from homogenates of liver, lung, spleen or kidneys. In the event that yeasts were cultured, the animal was not included in computation of the above data.

Footpad reactivity in cyclophosphamide pretreated mice

Both non-sensitized and sensitized, cyclophosphamide pretreated mice exhibited varying footpad responses when injected with heat-killed C. albicans as compared to non-drug treated controls (Table 7). Non-sensitized mice which received 200 mg/kg cyclophosphamide prior to challenge showed a similar response as non-treated controls. A maximum response was seen at 4 h with a reduction thereafter and a return to normal size by 48 h. The sensitized group that received 200 mg/kg cyclophosphamide showed a very different response. Whereas the nontreated sensitized group showed a maximum response at 24-48 h the cyclophosphamide pretreated mice showed a mild reaction at 4 h with an increased response at 24, 48 and 72 h. The foot did not return

Table 7. Footpad reactivity in cyclophosphamide (CY) pretreated non-sensitized and sensitized mice after injection of 2×10^5 heat-killed C. albicans.

	Net footpad response in recipients[a]				
Experimental	0 h	4 h	24 h	48 h	72 h
Non-sensitized	0 ± 0	33 ± 10	16 ± 8	5 ± 6	1 ± 5
CY-200 mg/kg[b]	0 ± 0	31 ± 4	3 ± 2	2 ± 3	1 ± 4
Sensitized[c]	0 ± 0	61 ± 14	105 ± 17	61 ± 20	17 ± 4
CY-200 mg/kg	0 ± 0	20 ± 2	37 ± 4	40 ± 7	50 ± 2

[a]mean increase in footpad thickness ± standard deviation in 0.01 mm; six animals per group.

[b]mice received a single intraperitoneal injection of cyclophosphamide 3-5 days prior to challenge.

[c]mice were sensitized with 4 weekly subcutaneous injections of 10^6 heat-killed C. albicans yeast cells.

to its preinjection size until 120 h post injection.

With cyclophosphamide pretreated mice, injection of live C. albicans produced a generally more intense footpad reaction over the course of the experiment than that seen in non-drug treated groups (Table 8). The 4 h response in non-sensitized mice which received 100 mg/kg of cyclophosphamide was slightly less than that seen in non-sensitized mice which had not received cyclophosphamide while the response seen in mice which had received 200 mg/kg of cyclophosphamide was approximately the same as the control group. By 24 h, the footpad reaction in the non-pretreated group showed a 50% decrease while those animals receiving cyclophosphamide exhibited a continued intense response. The 48 h reaction showed a decrease in both cyclophosphamide pretreated groups from the 24 h reading followed again by a slight decrease at 72 h. The non-pretreated mice showed similar 24 and 48 h responses followed by a decrease at 72 h. The inflammatory reaction was still greater at 72 h for the cyclophosphamide treated groups than seen in the non-treated mice.

The sensitized, cyclophosphamide pretreated groups showed less rapid increased 4 h and 24 h responses when compared to the sensitized mice which had not received cyclophosphamide. By 48 h, the reaction in this non-drug treated group was on the decline, while that seen in the cyclophosphamide pretreated groups showed a continued increase, more marked in the group that received 200 mg/kg of the drug. This increased response remained at 72 h for the 200 mg/kg group compared

Table 8. Footpad reactivity in cyclophosphamide (CY) pretreated non-sensitized and sensitized mice after injection of 2×10^5 live C. albicans.

	Net footpad response in recipients[a]				
Experimental	0 h	4 h	24 h	48 h	72 h
Non-sensitized	0 ± 0	101 ± 11	49 ± 10	51 ± 10	32 ± 10
CY-100 mg/kg[b]	0 ± 0	80 ± 13	107 ± 10	81 ± 6	72 ± 9
CY-200 mg/kg	0 ± 0	109 ± 15	100 ± 12	88 ± 10	90 ± 5
Sensitized[c]	0 ± 0	129 ± 12	131 ± 10	101 ± 16	41 ± 17
CY-100 mg/kg	0 ± 0	85 ± 17	103 ± 18	122 ± 12	93 ± 11
CY-200 mg/kg	0 ± 0	96 ± 15	106 ± 20	140 ± 20	137 ± 15

[a]mean increase in footpad thickness ± standard deviation in 0.01 mm; six animals per group

[b]mice received a single intraperitoneal injection of cyclophosphamide 3-5 days prior to challenge.

[c]mice were sensitized with 4 weekly subcutaneous injections of 10^6 heat-killed C. albicans yeast cells.

to a decreased response in the 100 mg/kg group. The sensitized mice which had not received the drug showed a gradual return to normal.

Overall, these results indicate an enhanced footpad response in both non-sensitized and sensitized mice, when pretreated with cyclophosphamide and injected with live C. albicans. This response was, in general, more pronounced in those mice which had received 200 mg/kg of cyclophosphamide in contrast to the 100 mg/kg groups and of longer duration. The footpad response was much greater in mice sensitized with heat-killed yeast cells both non-drug treated as well as drug treated than in mice not sensitized to C. albicans.

Viable cell count results in footpads of mice pretreated with cyclophosphamide are shown in Table 9. By 4 h, the number of viable cells had not decreased significantly in any of the cyclophosphamide pretreated animals. At 24 h, mice which had received 100 mg/kg of cyclophosphamide displayed a moderate decrease in live cells while those mice which had received 200 mg/kg of the drug demonstrated an increase in viable cell number. At 48 and 72 h, this trend continued with the 100 mg/kg groups exhibiting a steady decrease, but not as dramatic as in those animals not receiving cyclophosphamide. The 200 mg/kg groups displayed an increase in viable yeast cells through 72 h (Table 9). Cyclophosphamide pretreatment, seems to substantially reduce the ability to control the localized infection. Sensitized and non-sensitized mice, both with and without cyclophosphamide pretreatment, had no difference in the number of yeast cells present during the 72 h period.

Table 9. Viable yeast cells present foot homogenate of cyclophosphamide (CY) pretreated non-sensitized and sensitized mice after injection with 2 x 10^5 live C. albicans.

	CFU/foot at hours post-injection[a]				
Experimental	0 h	4 h	24 h	48 h	72 h
Non-sensitized	5.2 ± .05	4.7 ± .13	4.1 ± .15	3.3 ± .09	3.1 ± .05
CY-100 mg/kg[b]	5.2 ± .01	5.2 ± .01	4.9 ± .25	4.5 ± .08	3.6 ± .46
CY-200 mg/kg	5.2 ± .01	5.2 ± .16	5.8 ± .23	5.3 ± .03	5.3 ± .04
Sensitized[c]	5.2 ± .04	5.1 ± .14	4.2 ± .06	3.4 ± .25	3.2 ± .07
CY-100 mg/kg	5.2 ± .02	5.1 ± .06	5.0 ± .17	4.7 ± .10	3.5 ± .25
CY-200 mg/kg	5.2 ± .04	5.3 ± .05	6.0 ± .02	5.5 ± .02	5.3 ± .02

[a]mean $\log_{10}$ colony forming units (CFU) ± standard deviation; 5 animals per data point.

[b]mice received a single intraperitoneal injection of cyclophosphamide 3-5 days prior to challenge.

[c]mice were sensitized with 4 weekly subcutaneous injections of 10^6 heat-killed C. albicans yeast cells.

In vitro phagocytosis and killing of *C. albicans* yeast cells by PMN from non-sensitized, sensitized, and cyclophosphamide pretreated mice.

One hour after addition of *C. albicans* yeast cells to mouse peritoneal PMN, many phagocytosed yeast cells were seen inside the PMN. The percentage of germ tube production by phagocytosed (intracellular), as well as extracellular yeast cells (Figure 1) in the different groups is shown in Table 10. The values observed between non-sensitized and sensitized mice were not statistically different ($p > 0.05$) while the percent germinating in either of the cyclophosphamide pretreated groups was statistically significant ($p < 0.001$) when compared to either of the groups which had not received the drug. There was no significant observable differences in phagocytic activity in any of the groups. The PMN from the cyclophosphamide (200 mg/kg) group were statistically ($p < 0.001$) less able to inhibit germ tube production than PMN from mice which had received cyclophosphamide (100 mg/kg). Virtually all of the extracellular yeast cells were seen to produce germ tubes.

The results of the experiment to determine if incubation of normal PMN in various concentrations of cyclophosphamide altered germ tube inhibition capability are shown in Table 11. There was no difference in germ tube inhibition between non-pretreated PMN and those pretreated with cyclophosphamide. Also, cyclosphosphamide had no effect on the ability of extracellular yeast cells to produce germ tubes (Table 11).

Figure 1. Germination of phagocytosed C. albicans after 1 h

A. Mouse phagocyte showing 2 phagocytosed yeasts (arrows). Note the lack of a germ tube. (Wright) x 1400.

B. Intracellular yeast (arrow) with germ tube initial. Note germ tubes produced by extracellular yeasts. (Wright) x 1400.

C. Intracellular yeast showing prominent germ tube (Wright) x 1400.

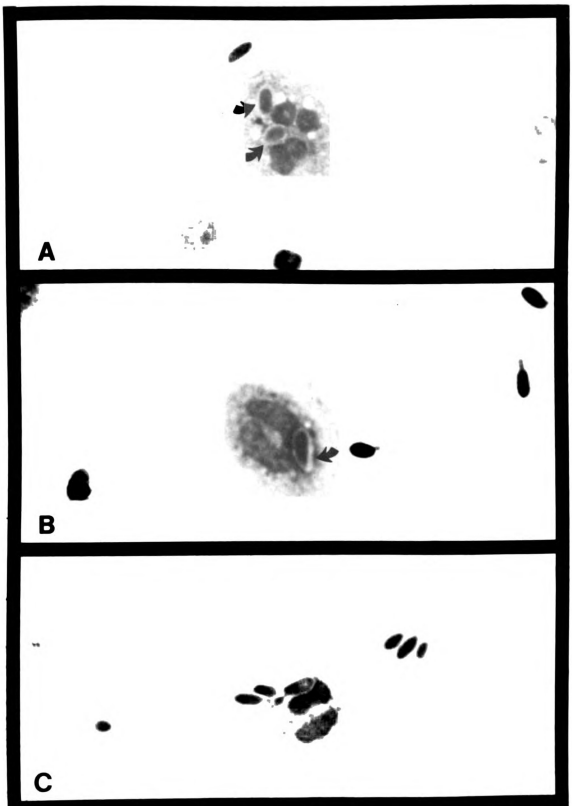
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Table 10. Germ tube production by C. albicans yeast cells phagocytosed by PMN of non-sensitized, sensitized and cyclophosphamide pretreated mice.

Experimental	% Germination[a] Intracellular	Extracellular
Non-sensitized	50 ± 2.8	99
Sensitized[b]	50 ± 1.7	99
Cyclophosphamide (100 mg/kg)[c]	71 ± 1.4[d]	99
Cyclophosphamide (200 mg/kg)	84 ± 2.9[d,e]	99

[a]mean percentage of 100 counted yeast cells producing germ tubes ± standard deviation.

[b]mice were sensitized with 4 weekly subcutaneous injections of 10^6 heat-killed C. albicans yeast cells.

[c]mice received a single intraperitoneal injection of cyclophosphamide 3-5 days prior to challenge.

[d]$p < 0.001$ vs. non-sensitized group.

[e]$p < 0.001$ vs. cyclophosphamide (100 mg/kg).

Table 11. Germ tube production by C. albicans yeast cells phagocytosed by PMN preincubated with cyclophosphamide.

	% Germination[a]	
Experimental	Intracellular	Extracellular
Normal PMN	50 ± 2.8	99
Cyclophosphamide pretreated PMN[b]		
0.01 mg/ml	46 ± 5	99
0.1 mg/ml	49 ± 7.2	99
1.0 mg/ml	47 ± 3.8	99

[a]mean percentage of 100 counted yeast cells producing germ tubes ± standard deviation.

[b]PMN were incubated for 1 h in the various concentrations of cyclophosphamide before addition of yeast cells.

Table 12. Killing of C. albicans yeast cells in the presence of PMN from non-sensitized, sensitized and cyclophosphamide pretreated mice.

Experimental	% Recovered	% Killed
Non-sensitized	56 ± 8[a]	44 ± 8
Sensitized[b]	58 ± 5	42 ± 5
Cyclophosphamide (100 mg/kg)[c]	78 ± 4[d]	22 ± 4
Cyclophosphamide (200 mg/kg)	90 ± 6[d]	10 + 6

[a]percentage ± standard deviation.

[b]mice were sensitized with 4 weekly subcutaneous injections of 10^6 heat-killed C. albicans yeast cells.

[c]mice received a single intraperitoneal injection of cyclophosphamide 3-5 days prior to challenge.

[d]$p < 0.001$ vs. non-sensitized group

Table 13. Killing of C. albicans yeast cells by PMN preincubated with cyclophosphamide.

Experimental	% Recovered	% Killed
Normal PMN	56 ± 8[a]	44 ± 8
Cyclophosphamide pretreated PMN[b]		
0.01 mg/ml	58 ± 5	42 ± 5
0.1 mg/ml	55 ± 7	45 ± 7
1.0 mg/ml	56 ± 6	44 ± 6

[a]percentage ± standard deviation.

[b]PMN were incubated for 1 hr in the various concentrations of cyclosphosphamide before addition of yeast cells.

The in vitro ability of mouse PMN to kill C. albicans yeast cells was assessed. Yeasts and PMN at a ratio of 1:10 were incubated for 2 h, then the number of viable yeast cells remaining in the medium was determined. The results (Table 12) showed no difference in the ability of PMN from non-sensitized and sensitized mice to kill C. albicans. Pretreatment of the mice with cyclophosphamide impaired this killing ability. PMN from mice receiving 100 mg/kg of cyclophosphamide showed better killing ability than those from mice which had received 200 mg/kg.

As with the phagocytosis assay, PMN from non-pretreated mice were exposed to cyclophosphamide. Results from this experiment showed no suppression of candidacidal activity (Table 13) in cyclophosphamide PMN when compared to normal PMN.

Footpad histopathology in non-sensitized, sensitized, and cyclophosphamide pretreated mice injected with live C. albicans.

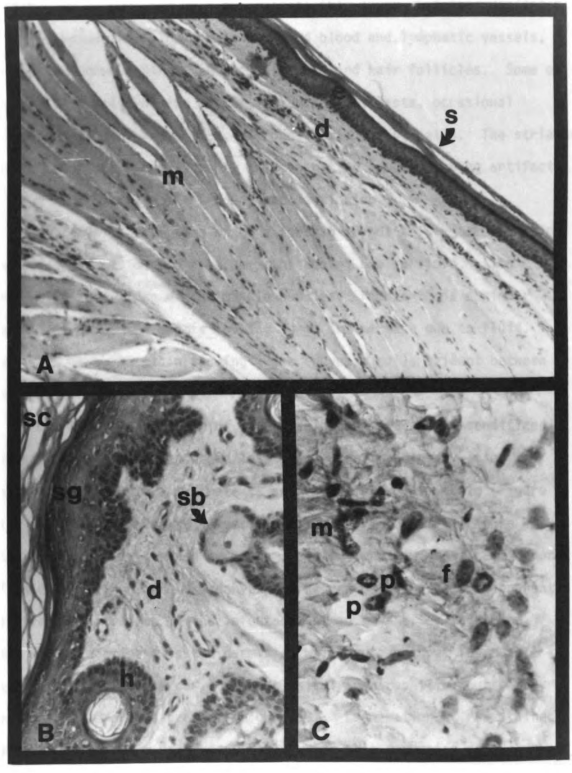
The histological section from a normal, uninjected mouse footpad is shown in Fig. 2. The outer desquammating layer of the epidermis, the stratum corneum, consists of keratinized tissue which is sloughed off during normal wear. Immediately below the stratum corneum are: the stratum lucidum and the stratum granulosum, the latter being more prominent and consisting of 2 or 3 layers of somewhat flattened cells containing granules of keratohyalin and eleiden. The innermost layer of the epidermis is the stratum spinosum, also called the stratum Malpighii or the germinal layer. Below the epidermis is the corium or

Figure 2. Histopathological section of a mouse footpad.

A. Sectioned mouse footpad showing stratum corneum (s), epidermis (e), dermis (d) and muscle (m). (H + E) X 140

B. Higher magnification showing stratum corneum (sc), stratum granulosum (sg), dermis (d), sebaceous gland (sb) and hair follicle (h). (H + E) X 280.

C. Resident cells of footpad dermis including mast cell (m), polymorphonuclear leukocyte (p) and fibroblast (f). (H + E) X 630.


s
d
m
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dermis. It is composed of a superficial thin layer that interdigitates with the epidermis. The dermis contains blood and lymphatic vessels, nerves and nerve endings, sebaceous glands and hair follicles. Some of the resident cells of the dermis include: fibroblasts, occasional polymorphornuclear leukocytes, macrophages, and mast cells. The striated muscle bundles are easily seen, the separation probably being artifactual due to dehydration, embedding and sectioning.

Figure 3A shows a section of a footpad immediately after injection. The epidermis and dermis have some thickening due to fluid. Also evident is separation of the muscle bundles. Figure 3B is a higher magnification of the dermis showing edema or increase due to fluid. Figure 3C is a PAS stain showing injected yeast cells evident between the muscle bundles.

Four h after injection of live C. albicans into a non-sensitized mouse footpad, the footpad had less edema than immediately after injection along with a moderate to severe cellular infiltrate (Figure 4A). This infiltrate was almost exclusively neutrophilic (Figure 4B). Examination of PAS stained sections revealed very few yeast cells, none of which were producing pseudohyphae. Figure 5A shows a more apparent cellular infiltration of the infected footpad 24 h after injection. Many PMN were present in the dermis as well as throughout the muscle layer. The infiltrate was still exclusively neutrophilic, but some eosinophils were also present (Figure 5B). Examination of PAS stained sections reveal very rare yeast cells which were producing pseudohyphae. These were seen within areas of localized severe cellular infiltration

Figure 3. Section of a mouse footpad immediately after injection with 2×10^5 live C. albicans.

A. Mouse footpad showing moderate dermal edema (arrows) and separating muscle fibers. (H + E) X 140.

B. Higher magnification of dermal edema. (H + E) X 630.

C. Injected yeast cells between muscle bundles (PAS) X 630.

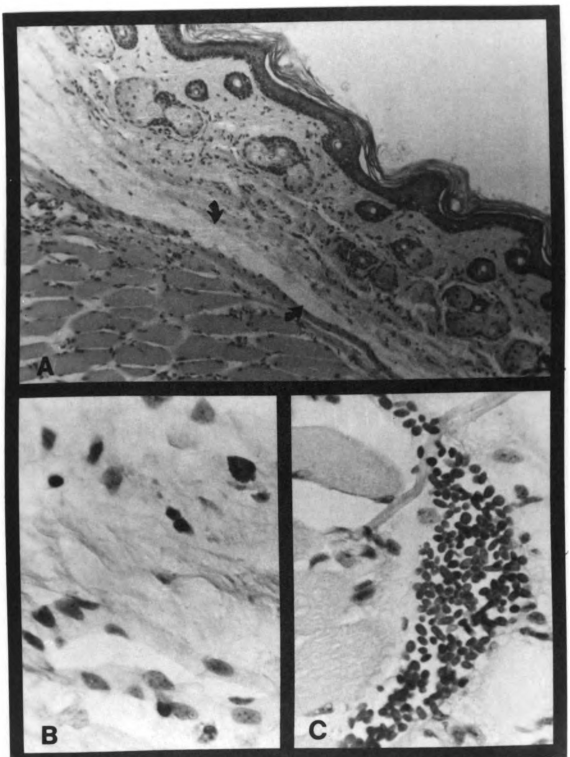
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Figure 4. Section of a non-sensitized mouse footpad 4 h after injection with 2 X 10^5 live C. albicans.

A. Mouse footpad showing some edema along with a moderate to severe cellular infiltrate. (H + E) X 140.

B. Cellular infiltrate showing an exclusively granulocytic component. (H + E) X 630.

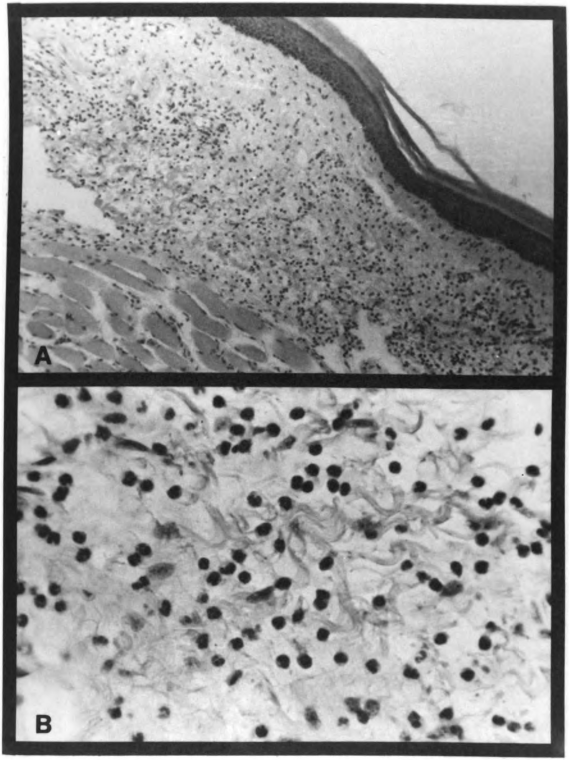
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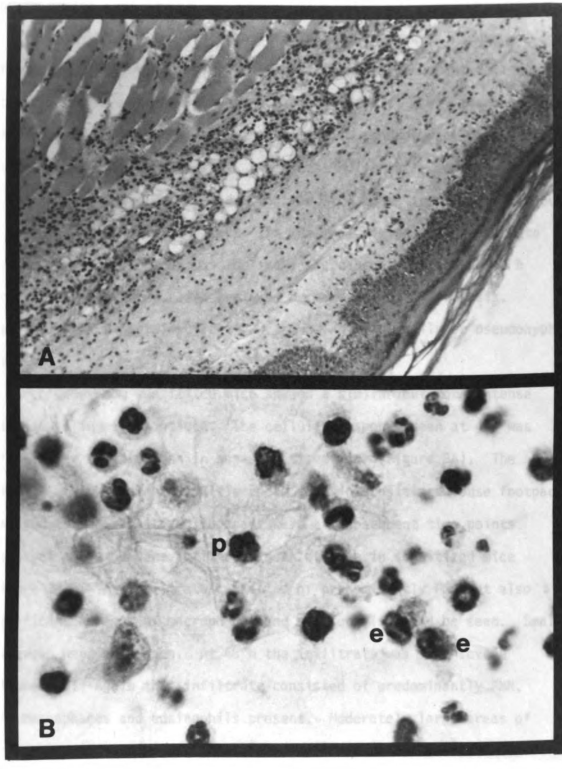
Figure 5. Section of a non-sensitized mouse footpad 24 h after injection with 2 X 10^5 live C. albicans.

A. Footpad section showing a moderate to severe cellular infiltrate, more concentrated between the dermis and the muscle. (H + E) X 140.

B. Cellular infiltrate showing mostly PMN (p) along with some eosinophils (e). (H + E) X 1400.


A
p
e
e
B

microabscesses (not shown).

Forty-eight h after injection, footpads showed very intense inflammation (Figure 6A). The PMN was still the predominant cell type but small numbers of macrophages were also evident. Small microabscesses were apparent (Figure 6B) and yeast cells with pseudohyphae were usually associated with these abscesses. The yeast cells were, however, rare in these areas. Figure 7 shows a footpad 72 h after injection. Although an infiltrate was still apparent, the foot was beginning to return to its preinjection appearance. The small microabscesses seen at 48 h were no longer evident being replaced by early signs of fibrosis. Examination of PAS stained sections showed no yeast cells or pseudohyphae present.

Footpads from sensitized mice showed a similar but more intense response to injected antigen. The cellular response seen at 4 h was very similar to that seen in non-sensitized mice (Figure 8A). The greatest histopathological differences seen in sensitized mouse footpads compared to non-sensitized footpads were at subsequent time points. There was a more severe infiltrate seen at 24 h in sensitized mice (Figure 8B). The infiltrate consisted of predominantly PMN but also significant numbers of macrophages and eosinophils could be seen. Small abscesses were also seen. At 48 h the infiltrate was very severe (Figure 9A). Again this infiltrate consisted of predominantly PMN, with macrophages and eosinophils present. Moderately large areas of very severe infiltrate with associated necrosis were observed, with rare PAS positive yeast cells present. Some early fibrosis was also

Figure 6. Section of a non-sensitized mouse footpad 48 h after injection with 2 X 10^5 live C. albicans.

A. Mouse footpad showing severe cellular infiltrate. (H + E) X 140.

B. Small microabscess. (H + E) X 630.

A
B

Figure 7. Section of a non-sensitized mouse footpad 72 h after injection with 2 X 10^5 live C. albicans.

A. Mouse footpad showing mild residual cellular infiltration. (H + E) X 140.

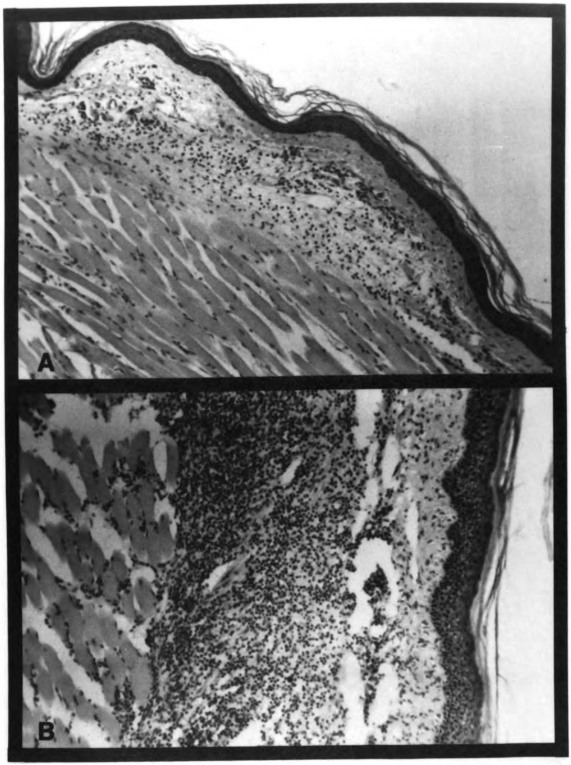
B. Evidence of early fibrosis (arrows). (H + E) X 630.

A
B

Figure 8. Footpad sections of sensitized mice after injection with 2×10^5 live C. albicans.

A. 4 h after injection. Moderate cellular infiltrate, some edema. (H + E) X 140.

B. 24 h after injection. Severe infiltrate, layer of cellulitis is apparent adjacent to muscle. (H + E) X 140.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B

Figure 9. Footpad sections of sensitized mice after injection with 2×10^5 live C. albicans.

A. 48 h after injection. Very severe infiltration with associated necrosis can be seen. (H + E) X 140.

B. 72 h after injection. Very severe infiltrate. (H + E) X 140.

A
B

evident. Sections at 72 h post-injection still revealed a very heavy infiltrate (Figure 9B) with more macrophages present.

Footpads from mice, both non-sensitized as well as sensitized, pretreated with 200 mg/kg of cyclophosphamide also exhibited a cellular response when injected with a suspension of live C. albicans yeast cells. This response was not nearly as intense as that seen in animals not pretreated with the drug. In addition, large areas of necrosis had developed and much yeast proliferation was evident in these areas. Figure 10A shows the moderate edema and mild cellular infiltrate seen in a non-sensitized, cyclophosphamide pretreated mouse footpad 4 h after injection with live C. albicans. Figure 10B shows an area of the dermis within the footpad and the proliferating yeast cells and pseudohyphae are apparent in this area (Figure 10C). The cellular infiltrate was composed of predominantly PMN. Twenty-four h after injection, the inflammation seen was more severe than at 4 h (Figure 11A) although not as intense as that seen in non-cyclophosphamide pretreated mice. In addition, more macrophages seen in the infiltrate than were seen in mice that had not received cyclophosphamide. As with the 4 h sections, some microabscesses with yeast cells and pseudohyphae were present. The 48 h sections showed more foci of infection as well as severe muscle damage (Figure 11B). Many macrophages were present in the infiltrate. At 72 h (Figure 11C) the infiltrate consisted primarily of macrophages along with degenerating PMN. Large abscesses were evident and examination of PAS stained sections showed many yeast cells

Figure 10. Footpad sections of non-sensitized, cyclophosphamide pretreated mice 4 h after injection with 2 X 10^5 live C. albicans.

A. Mouse footpad showing dermal edema and moderate cellular infiltrate (H + E) X 140

B. Area showing fungal proliferation (PAS). X 140

C. Yeast cells and pseudohyphae in tissue space. (PAS) X 630.

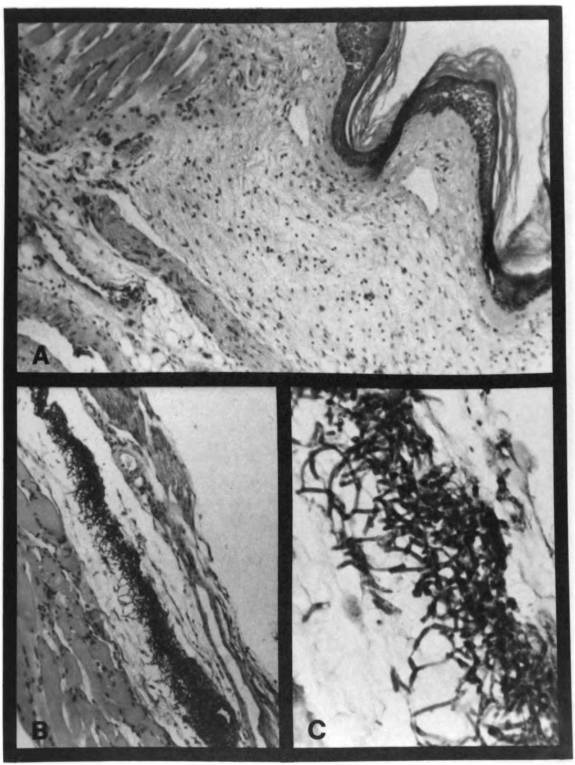
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Figure 11. Footpad sections of non-sensitized, cyclophosphamide pretreated mice after injection with 2 X 10^5 live *C. albicans*.

A. Mouse footpad 24 h after injection. Severe infiltrate (H + E) X 140

B. Mouse footpad 48 h after injection. Severe infiltrate and much muscle damage. (H + E) X 140

C. Mouse footpad 72 h after injection. Large abscess with necrotic center can be seen. (H + E) X 140.

A
B
C

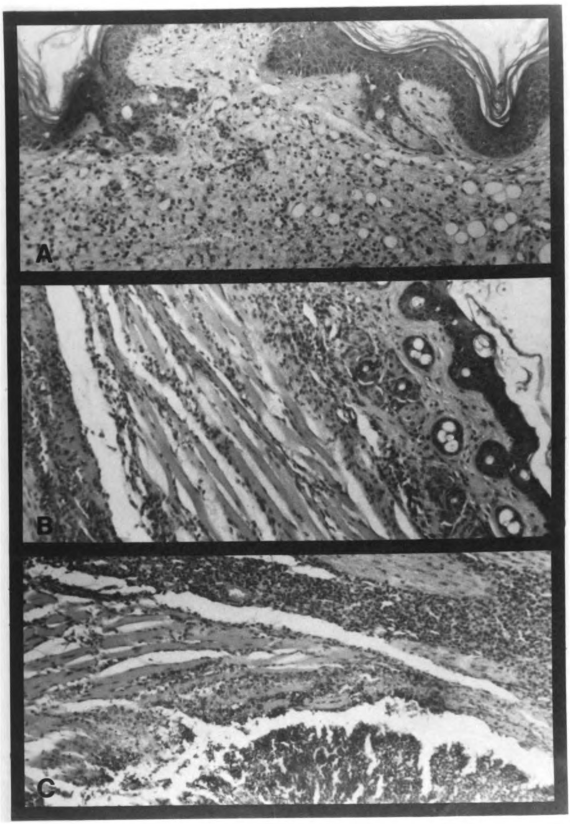
with pseudohyphae. Sensitized, cyclophosphamide pretreated mice showed more cellular infiltration than was seen in non-sensitized, drug treated animals (Figure 12). As with the non-sensitized, cyclophosphamide pretreated mice, large areas of focal suppurative inflammation had formed by 48 h. Severe muscle damage was evident and large aggregates of yeast cells and pseudohyphae were seen in these areas. Macrophages did, however, comprise a significant portion of the cellular infiltrate at 24 h, more so than in non-sensitized, cyclophosphamide pretreated mice.

Figure 12. Footpad sections of sensitized, cyclophosphamide pretreated mice after injection with 2 X 10^5 live C. albicans.

A. Mouse footpad 24 h after injection. Severe inflammation consisting of PMN and macrophages. (H + E) X 140.

B. Mouse footpad 48 h after injection. Severe inflammation, much muscle damage. (H + E) X 140

C. Mouse footpad 72 h after injection. Cellular infiltrate mostly macrophage with degenerating PMNs. (H + E) X 140. Part of an abscess is seen at bottom of picture.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DISCUSSION

The observation that infections by C. albican in healthy hosts are very rarely encountered seems to indicate that non-specific host defense mechanisms greatly contribute to resistance to infection. It is only when this system is compromised, either naturally, experimentally or the unfortunate result of treatment for some other disorder that infection by this organism will proceed. In addition, a majority of healthy adults show positive antibody and/or cell-mediated hypersensitivity responses to C. albicans which indicate that specific acquired immunity may play a role in defense from infection.

Over the years, various animal models of candidosis, representing the diverse clinical pictures presented by these infections have been developed (Rogers and Balish, 1980). They include models for cutaneous, vaginal and disseminated disease. Experimental manipulation of these models, usually by means of pretreatment with agents which suppress or activate innate or acquired defense systems, have led to a greater understanding of host-parasite interactions regarding this organism.

Of particular interest is the in vivo footpad microbicidal technique first described by Shepard in 1960. This self-limiting localized infection was developed to follow intralesional populations of an obligate parasite, Mycobacterium leprae, and in assessing various chemotherapeutic agents used in the treatment of leprosy. The mouse footpad swelling test for detection of hypersensitivity, especially of the delayed type, has been utilized extensively (Crowle, 1975). Rifkind et al. (1976) have adapted this model for use with C. albicans.

By combining features of the reported uses of the model, i.e. quantitative intralesional population changes and hypersensitivity responses along with histopathological examination of infected footpads, a better understanding of intralesional events after infection could be realized. In addition, experimental manipulation of the host prior to challenge could contribute more information regarding the infectious process. The experimental strategies utilized in this research consisted of following the course of a self-limiting fungal infection in normal, immunologically hypersensitive and metabolically compromised mice. Additionally, *in vitro* experiments were used to help clarify *in vivo* observations.

Immune responses in animals sensitized with heat-killed *C. albicans* yeast cells indicated that immunological hypersensitivity specific for this organism was induced. Precipitating anti-*C. albicans* antibody was present in serum and specific footpad swelling responses to injected antigen were seen. Sera from non-sensitized mice failed to show detectable anti-*C. albicans* antibody or positive footpad responses indicating little, if any, past exposure to *C. albicans*.

Precipitating antibodies have been reported in mice previously sensitized with live or heat-killed *C. albicans* (Giger *et al.*, 1978; Evron, 1980). The total quantity of yeasts used in the sensitization procedures correlated well with the percentage of positive sera. Giger *et al.* (1978), using counterimmunoelectrophoresis, demonstrated precipitins in 92.3% of sera from mice sensitized twice with 10^6 live

C. albicans yeast cells and 54% of mice inoculated twice with 5×10^5 yeasts. Only 10% of mice inoculated twice with 5×10^5 heat-killed yeasts showed precipitating antibodies. In the present study, when mice were sensitized with a total of 4×10^6 heat-killed yeast cells all sera tested positive for precipitins. A larger number of organisms inoculated each time would probably favor greater antibody production (Davis et al., 1973). The inoculation of smaller numbers of viable cells could contribute to greater antibody production due to the less rapid degradation and hence, a longer duration of stimulus for antibody production. Evron (1980) reported precipitating antibodies present in sera from mice sensitized once i.p. with 4×10^6 live C. albicans suspended in saline or with s.c. injection of 6×10^6 to 8×10^7 heat-killed yeasts suspended in Freund's complete adjuvant (FCA). Since the sera from these groups of mice were pooled, the percentage of those positive for precipitins was not reported. In both of the above reported studies, sera from mice not sensitized showed no detectable precipitins.

No anti-C. albicans precipitating antibodies were detected in mice sensitized with C. krusei antigen using oidiomycin, a C. albicans soluble extract. The lack of anti-C. albicans antibody could be explained by the investigation of Sweet and Kauffman (1970). They showed that of the 13 antigens recognized in medically important species of Candida, C. albicans and C. krusei have only 2 common antigenic determinants.

Agglutinating antibodies were not detected in any of the mouse sera tested. This result is in agreement with those reported by Giger et al. (1978) but contrary to the report of Evron (1980). Specific anti-C. albicans agglutinating antibodies have been demonstrated in other mammalian systems, specifically rabbit (Sweet and Kauffman, 1970), human (Rippon, 1974) and cow (see Materials and Methods, Preparation of bovine anti-C. albicans antiserum). Mouse serum has been reported to agglutinate bacteria (Friedman and Moon, 1980).

One possible explanation for the results of Evron (1980) is her use of FCA, a potent antibody production stimulator (Davis et al., 1973). The possibility exists that continued production of antibodies of the IgM class, shown to be better agglutinins, contributed to these results. The problem of disparate results in agglutinating activity of mouse sera and yeast cells deserves further attention.

Strong footpad reactivity was seen in all mice tested which had been sensitized with heat-killed yeasts and challenged with the same antigen (Table 1). At all times after injection footpad responses were significantly greater in the sensitized group when compared with the non-sensitized group. Rifkind et al. (1976) were the first to adapt the mouse footpad swelling test for use with fungal antigens. Their results, utilizing heat-killed C. albicans are very similar to the results obtained in this study at 48 h post-injection. They did not, however, indicate swelling values for other time points as was reported in this study. Giger et al. (1978) and Evron (1980) also reported

footpad swelling values in mice sensitized with heat-killed C. albicans. In these two studies, though, the challenge antigen used was a soluble cytoplasmic extract whereas Rifkind et al. (1976) and this investigation, used heat-killed cells. Evron (1980) reported significant footpad responses at 4 h, peaking at 48 h and waning thereafter while Giger et al. (1978) showed little swelling at 4 h and none thereafter. As with the results in precipitating antibody production, the total amount of sensitizing antigen used in their procedure may not have been adequate to induce footpad reactivity.

Results of the present study show that the footpad response seen is specific for the sensitizing antigen (Table 2). Mice only reacted to the antigen with which they were sensitized and showed the same time sequence of reactivity. Collins and Mackaness (1968) previously reported the ability of the mouse footpad model to distinguish between different species of Salmonella. Rifkind et al. (1976) also showed specificity for the sensitizing antigen.

The small footpad swelling value seen in non-sensitized mice at 4 h can probably be attributed to the use of a particulate antigen for challenge (Table 1). The cause of the swelling was most likely non-specific inflammation due to the use of this antigen type, however the presence of undetectable amounts of circulating antibody to yeasts that contributed to this reactivity cannot be discounted. Mice may normally harbor yeasts in their gastrointestinal tract, and these yeasts may have common antigens with the challenge yeast (Savage and Dubos, 1967).

No increase in footpad size attributable to trauma from injection was noted.

Allergic inflammation seen at 4 h post-injection is usually referred to as an Arthus reaction, or immediate type hypersensitivity (ITH), while inflammation that takes 24 to 48 h to peak is termed delayed type hypersensitivity (DTH). The results presented in this study for sensitized mice show a strong 4 h response peaking at 24 h and waning thereafter. It is not unreasonable to postulate that both types of hypersensitivity reactions are seen, an initial antibody-mediated ITH followed by an overlapping DTH. Alternately, the reaction could be thought of as a prolonged ITH. Due to continued presence of the particulate antigen *in situ*, antibody may be produced locally and therefore contribute to an inflammation due to continued production of opsonins (e.g. complement factors C3a and C5a).

Rifkind *et al*. (1976, 1977) showed that footpad reactivity to *C. albicans* could be transferred to a naive animal via sensitized spleen cells or Lawrence-type transfer factor and not with immune serum. Evron (1980) demonstrated the presence of macrophage migration inhibition factor (an *in vitro* indicator of cell-mediated hypersensitivity) in mice sensitized with either live or heat-killed *C. albicans* cells. In the experiments reported by these investigators, the footpad reactions were maximal at 24 to 48 h and were therefore classified as delayed hypersensitivity responses. Based on the results of these published reports, it is reasonable to assume that the footpad reactions seen in

the present study are of the delayed type although they probably contained a strong Arthus component as suggested by the presence of circulating antibodies as well as a significant 4 h response.

Footpad reactivity in non-sensitized and sensitized mice after injection of viable C. albicans was similar to that seen with heat-killed yeast cell challenge except the response was much more enhanced (Tables 1 and 5). Again, a much greater response was seen in sensitized mice than in the non-sensitized group indicating the response was at least in part, immunologically based. Three possibilities can be postulated for the greater response seen in mice injected with live cells as compared to heat-killed cells. 1) An increase in fungal cell load due to multiplication of the cells after injection, 2) the production of a toxin by actively metabolizing yeast cells in tissue, or 3) pyrogens excreted as a result of cell metabolism or present on the cell surface. Although these pyrogens cannot be considered toxins, they do have toxic properties and thereby can increase inflammation.

The first possibility can be discounted as shown by the results presented in Table 6. There was a significant decrease in the numbers of viable cells recovered from tissue 24 h after injection. At least 90% of the cells injected were not viable at this time and by 72 h greater than 99% of the injected cells were killed. There was no difference in the ability of the animals of either group, non-sensitized or sensitized, to control the infection. There was no dissemination of the yeast cells from the foot to other parts of the body at any time as proven by negative culture results of homogenized organs.

Oghiso and Matsuoka (1979) showed, that after injection of colloidal carbon into mouse hind footpads, there was very slight egress of the particles to other areas of the body. Essentially, all of the suspension remained in the footpad or was trapped in the popliteal lymph node. Since carbon particles are much smaller in size than intact yeast cells, it can be assumed that the injected yeasts remained at or near the site of injection. Care was taken, however, to include the area containing this lymph node when the foot was excised and homogenized.

The second possibility, the production of a toxin by C. albicans is a controversy as evidenced by the many published reports. This was reviewed by Odds (1979). He concluded that there is no substantial evidence to suggest that exotoxins, analagous to the toxins secreted by bacterial pathogens, are produced by C. albicans or other yeasts.

The third possibility seems more tenable. Candida surface glycoproteins have pyrogenic properties. Cutler et al. (1972) obtained pyrogenic reactions in rabbits which were elicited with whole cells and cell walls of C. albicans but not with cytoplasmic fractions. This pyrogenic activity was not seen when the preparations were heated previously. Holder and Nathan (1973) illustrated toxin-like properties i.e., aggregation of platelets in sonically disrupted viable C. albicans cells but none in disrupted heat-killed cells. One may conclude that the toxic effects seen in candidal infections are manifestations of the host response to intact viable cells of Candida or to some components of Candida released in the process of digestion by phagolysosomes.

Results presented here indicate that there was no added protection to lethal C. albicans challenge as a result of prior sensitization with heat-killed yeast cells. Both non-sensitized as well as sensitized mice showed approximately equal mortality after i.v. injection of viable C. albicans (Table 3).

Acquired immunity to infection with C. albicans in experimental animals has been the subject of many reports. Mourad and Friedman (1961) first showed some protection to lethal challenge in mice previously inoculated s.c. with either live, merthiolate-killed, or sonically disrupted C. albicans. Merthiolate-killed cells conferred the least protection while sonicated cells gave the best. No data was presented on the immune status of the mice i.e., the presence of antibody or skin reactivity at the time of challenge. Hasenclever and Mitchell (1963), using a different route of sensitization, i.p., and a dose of approximately 2×10^7 live yeast cells given at 14 and 7 days before challenge, showed increased survival. Intraperitoneal sensitization with heat-killed yeast cells suspended in incomplete Fruend's adjuvant conferred no protection. Again, the mice were not assessed for specific immune responses. Giger et al. (1978) showed that some protection could be seen in mice previously sensitized s.c. with either live or heat-killed cells compared to those not previously exposed to Candida. This protection was short-lived as mice sensitized 2 weeks before i.v. challenge showed protection while those sensitized 4 weeks before challenge were not protected.

Other investigators, using non-specific immune activators, have attempted to show protection to lethal C. albicans challenge. Marra and Balish (1974), using mice sensitized with Listeria monocytogenes, reported short-lived immunity to C. albicans challenge as measured by faster elimination of viable yeast cells from various organs. Rogers and Balish (1977) showed no protection to C. albicans challenge in mice vaccinated with Mycobacterium bovis (strain BCG) but increased protection against L. monocytogenes infection.

The literature cited above show that a variety of experimental strategies have been used in attempts to confer protection to lethal challenge with C. albicans. These attempts have resulted in limited success and if protection was induced it was of short duration. Collins (1974) has criticized these "protection studies" used as a basis for development of vaccines. Increases in the mean time to death have very little relevance if the majority of both vaccinated and control groups eventually succumb to the challenge infection. Thus, he has suggested comparing the fate, either elimination or multiplication, of the challenge organism in vaccinated and control groups.

There seems to be some confusion in the literature concerning the use of certain terminology, specifically immunization, vaccination and sensitization. Immunization refers to the process or procedure by which resistance to microbial challenge is conferred as the result of a previous inoculation. For instance, laboratory mice inoculated with M. leprae resulted in resistance to infection by L. monocytogenes and

M. tuberculosis (Patel and Lefford, 1978) as well as Salmonella enteriditis (Collins, 1974). Vaccination, on the other hand, refers to the injection of a suspension of a specific microbe (or some part of it) as a means of prophylaxis or cure of the disease caused by that organism. An example is the immunity to pertussis (whooping cough) after inoculation with either killed Bordetella pertussis bacteria or of a bacterial fraction. Sensitization refers to the process by which an individual has been rendered susceptible to immunological reactions by previous exposure of the immunological system to the antigens concerned. These reactions may not be associated with resistance to infection. In the present study, it seems justified to use the term "sensitized". Animals previously inoculated with heat-killed C. albicans show hypersensitivity after antigen exposure as evidenced by specific circulating antibody and strong footpad reactions (Table 1) but no acquired immunity to lethal challenge (Table 3).

Pretreatment of mice with cyclophosphamide (CY) greatly increased their susceptibility to C. albicans infection (Table 3). This finding is in agreement with published reports using other infectious agents. Robinson et al. (1969) showed that the nonlethal infection of mice with Sendai virus was converted to a fatal pneumonic illness when CY was administered while Hurd and Heath (1975) reported similar results using mice infected with influenza virus. Moser and Domer (1980) reported that the net effect of CY treatment was to nullify any ability of mice to develop resistance to reinfection. As in the present study, they

also reported a significant increase in mortality after i.v. injection of C. albicans in CY pretreated mice.

An increased susceptibility to infection implies some alteration in resistance mechanisms of the host. The possibility that this alteration is single in nature seems remote. It is more probable that a multitude of factors are involved. Although numerous side effects of CY therapy occur such as hemorrhagic cystitis with hematuria and diarrhea, severe leukopenia is the most marked (Sharbaugh and Grogan, 1969). In the present study, all mice pretreated with CY showed significant depression in the numbers of peripheral white blood cells especially the PMN (Table 4). This cell type was significantly reduced in numbers in both drug-pretreated groups when compared to non-treated controls. No other side effects associated with CY pretreatment were observed in this study. Moser and Domer (1980), using 200 mg/kg of CY to pretreat mice also showed significantly lowered total leukocyte counts with the reduction in numbers of PMN being the most marked.

Mice pretreated with 200 mg/kg of CY showed an even greater suppression of PMN than those which received 100 mg/kg of the drug (Table 4). Sharbaugh and Grogan (1969) reported a similar dose dependent leukocyte suppression in rats. By increasing or decreasing the dosage of CY used to pretreat the animals, the severity of peripheral leukopenia could be controlled.

Although the exact mode of action of CY is not known, it seems likely that the drug inhibits the incorporation of precursors into DNA

(Hill, 1975). Since a clinical use of CY is to arrest the growth of tumor cells, inherently rapidly dividing cells, this explanation seems plausible. It is an unfortunate side effect of CY therapy that normally rapidly dividing cells such as bone marrow stem cells are also affected. Joyce and Chervenick (1977) observed that the number of marrow progenitor cells was maximally depressed in mice 3-5 days after CY treatment as was the number of peripheral blood PMN. For this reason, mice in the present study were used experimentally at least 3 and no longer than 5 days after CY pretreatment.

The results discussed so far for CY pretreated mice show several advantages for its use in studying infectious diseases. 1) Cyclophosphamide pretreatment results in selective depletion of white cells of the granulocyte type, in addition, this effect appears to be dose dependent although at high doses, significant reduction of all leukocyte types is seen (Table 4). 2) Increased susceptibility to infection with relatively non-virulent microorganisms can be induced. 3) There appears to be a lack of noticeable side effects after drug treatment. It is also suggested that the PMN is of prime importance in resistance to infection with C. albicans since depletion of this cell type correlated well with mortality.

The footpad swelling responses seen after injection of heat-killed yeast cells in CY pretreated non-sensitized mice showed a similar response as that seen in non-treated controls (Table 7). The response seen in the CY pretreated sensitized group was of lesser intensity than that of non-treated sensitized mice. The response seen appeared to be

immunologically based since it was greater than that of the non-sensitized CY pretreated group. This indicates that sensitized mice pretreated with CY, even at the largest dosage, still retain immunological reactivity albeit not as pronounced as in non-drug treated sensitized mice. In addition, the ability to mount an immediate inflammatory response, as evidenced by the 4 h swelling increase, is not affected by drug treatment.

The effects of CY on immune reactions have been the subject of several reports. Cyclophosphamide appears to specifically affect B-cell function (Stockman et al., 1973) although T-cell suppression has been shown (Sharma and Lee, 1977). Beaman and Maslan (1977) reported a marked increase in susceptibility to Nocardia asteroides infections in mice after CY treatment. An intact humoral response is required for protection, from infection as well as recovery from the infection (Rippon, 1974). Mice were more susceptible to Histoplasma capsulatum infections after CY pretreatment (Cozad and Lindsey, 1974). Recovery from this infection is associated with the cellular (T-cell) component of the immune system (Rippon, 1974). Moser and Domer (1980), using CY pretreated live C. albicans sensitized mice, showed significantly reduced precipitating antibody production as compared to animals not treated with CY. They suggested that the ability to produce antibody at the time of challenge is crucial to defense against the infection. Based on the results of these publised reports, it seems that CY affects both components of the immune system although B-cell suppression seems to be the most pronounced.

The footpad swelling responses after injection of viable C. albicans observed in CY pretreated mice, both non-sensitized and sensitized, were greater in magnitude and of longer duration than seen in those mice not pretreated (Table 8). Mice pretreated with CY showed a dose dependent inability to control this footpad infection as evidenced by viable yeast cell counts from foot homogenates (Table 9). These counts seemed to correlate with the swelling observed. Thus the increased size may have been due, in part, to multiplication of the yeasts within the lesion.

Moser and Domer (1980), using a cutaneous C. albicans infection model, reported a similar inability of CY pretreated mice to control the numbers of injected organisms. They showed up to 100-fold increases in numbers of viable cells recovered from lesion homogenates 3 days after inoculation. There were larger lesion volumes after injection than in non-CY pretreated mice. Sensitized mice which received CY 3 days before cutaneous challenge had larger lesion volumes than similarly pretreated non-sensitized mice. There was no difference in the ability of non-sensitized or sensitized CY pretreated mice to clear the infection. Dissemination to other body organs increased as a result of CY pretreatment. No dissemination of inoculated yeasts was observed in the present study, utilizing the footpad model.

Examination of histopathological sections of mouse footpads revealed an acute inflammatory (granulocytic) cellular infiltrate soon after injection (4 h) of viable yeast cells. No discernible differences between the infiltrates of non-sensitized and sensitized mice were seen

at this time but differences were apparent at subsequent times. Sensitized mice showed a mononuclear component in the cellular infiltrate not seen in non-sensitized mice. The PMN did, however, retain numerical superiority throughout the entire experimental period in sensitized mice.

Histopathological responses in footpads of mice previously sensitized with heat-killed C. albicans and injected with the same antigen have previously been described (Rifkind et al., 1976). This response was characterized by a cellular infiltration consisting of neutrophils and mononuclear cells with the latter component being more intense at 48 h than at 24 h. Non-sensitized mouse footpads showed an exclusively neutrophilic response to the antigen. Surprisingly, they reported no swelling 4 h after antigen injection in either group. This is inconsistent with the results of the present study (Table 1). Both studies used the same antigen type (whole heat-killed yeast cells) as well as the same sensitization procedure. Injection of a particular suspension should result in a non-specific, but measurable, inflammatory response (Davis et al., 1973; Roitt, 1974). Additionally, the results of this study showed a significantly greater 4 h response in sensitized mice than seen in non-sensitized mice. This, coupled with the demonstration of specific anti-C. albicans antibody in sensitized mouse sera, suggests an immunological basis for this early swelling increase. Rifkind et al. (1976) did not evaluate their sensitized animals for humoral responses, i.e., antibody.

Histopathological responses to infection by C. albicans have been described for a number of animal models. Giger et al. (1978), in a study of the pathology induced by cutaneous infection with C. albicans, reported the cellular infiltrate to be composed of mixed acute and chronic inflammatory cells in and around areas of dermal abscess. Animals which were previously sensitized and then challenged showed a more intense infiltrate, of the same composition as that seen in non-sensitized mice. The histopathological reactions to live and dead yeasts were strikingly different. Abscesses frequently developed with the former, whereas no abscesses were seen with latter. Pearsall and Lagunoff (1974) reported in their mouse-thigh lesion model that the cellular infiltration consisted primarily of neutrophils one week after infection. At 2 weeks the infiltrate had increased in intensity and at that time, more macrophlages, eosinophils and lymphocytes were seen. In human infections, both cutaneous and systemic, the PMN is the largest component of the cellular infiltrate (Rippon, 1974; Rogers and Balish, 1980).

The results of footpad homogenization when combined with the histopathological observations suggest a correlation. As the intensity of the cellular infiltrate increases, the number of viable yeast cells correspondingly decreases. Twenty-four hours after injection the numbers of viable yeast cells remaining in the foot was approximately 10% of the original inoculum, or up to 90% of the injected cells had been killed. Hematoxylin and eosin stained footpad sections showed

at 24 h, in both non-sensitized and sensitized mice, was composed of predominantly PMN although macrophage were more evident in sections from sensitized mice. At 72 h after inoculation about 1% of the injected yeast cells were still viable in both groups. These data suggest that the PMN is the cell responsible for the elimination of the microorganism early in the infection.

Further evidence for the significance of the PMN is seen when results of CY pretreatment are analyzed. The histopathological responses observed, soon after infection, showed a much less intense cellular infiltrate. As early as 4 h after injection, areas of fungal proliferation within necrotic spaces were seen (Figures 10B and 10C). Similar necrotic areas were evident at subsequent times after 4 h. The corresponding viable yeast cell counts at the experimental times (Table 9) confirmed fungal multiplication, during the course of the infection.

Is the inability of CY pretreated mice to control this localized infection due to: 1) the smaller number of PMN available that can be recruited to combat this infection, 2) PMN being produced that are deficient in their ability to kill C. albicans, or 3) both of the above?

A series of in vitro experiments were designed to try to answer the above questions using suspension of mouse PMN and C. albicans. After incubation of PMN for non-drug treated mice with C. albicans, approximately 50% of the injested yeast cells produced germ tubes (Table 12). This indicated the other 50% were either dead or still

viable although inhibited in their germ tube production ability. The *in vitro* candidacidal assay was used in an attempt to determine if these non-germinated cells were viable. The results showed that approximately 57% of the yeasts were still viable after incubation of murine PMN and yeast suspension (Table 12). Thus, there seemed to be good correlation between inhibition of germ tube production and yeast cell death. Identical experiments using PMN from CY pretreated mice showed both the inability to prevent germ tube formation (Table 10) as well as decreased candidacidal activity (Table 12). By preincubating PMN from non-CY treated mice in various concentration of CY, these deficiencies were not evident (Tables 13 and 15). This suggests that the PMN being produced in CY pretreated mice may have altered candidacidal capabilities.

The actions of PMN on *Candida* have been extensively studied *in vitro*. These studies have been reviewed by Odds (1979) and Rogers and Balish (1980). The salient facts of these studies are as follows: 1) *C. albicans* yeast cells are rapidly phagocytosed *in vitro* by PMN, 2) some injested cells escape killing by producing germ tubes and/or pseudohyphae and break out of the phagocyte, and 3) this ability correlates well with the relative virulence for laboratory animals.

Sharbaugh and Grogan (1969) observed no significant changes in the injestive powers of phagocytes from animals pretreated with CY. They did observe striking decreases in the ability of phagocytes to kill injested *Pseudomonas aeruginosa*. Moser and Domer (1980) also suggested altered functional capabilities of PMN in their cutaneous model of

C. albicans infection. In addition, CY induces a significant decrease in the levels of fibrinolytic and proteolytic enzymes of leukocytes (Prokopowicz et al., 1967). It seems, therefore, that although a major side effect of CY therapy is severe leukopenia, the cells that are produced have altered function. A possibility exists that interaction between the phagocytes membrane and lysosomes of phagocytic cells is hindered in some manner, thus preventing lysosomal enzyme release and therefore altered killing capacity of the cell.

These investigations using the footpad model have shown that the PMN is the primary phagocytic cell responsible for the defense against infection by C. albicans. Induction of specific ITH and/or DTH seem to have no effect on the elimination of the infectious agent. Cyclophosphamide therapy seems to not only depress PMN production but also PMN function. In addition, the footpad model as presented here, can be further developed as an experimental tool to better understand basic host-parasite interactions and the forces which decide infection or health of the host.

SUMMARY

This study characterized the host response to a self-limiting C. albicans infection. Groups of mice, non-sensitized, sensitized and CY pretreated, were injected via a hind footpad with viable yeast cells. Footpad swelling responses, numbers of viable yeasts remaining in the lesion and histopathological examination of thin sections were evaluated over the course of the infection.

Non-sensitized mice exhibited maximal 4 h footpad swelling responses after injection with a waning thereafter. By 72 h post-injection, the foot had essentially returned to its pre-injection size and approximately 99% of the injected yeasts had been killed. A cellular infiltrate composed of PMN with few eosinophils was seen.

Mice previously inoculated with heat-killed C. albicans showed circulating anti-C. albicans antibody as well as a specific footpad swelling response to injected antigen. This footpad response had immediate as well as delayed hypersensitive characteristics. There was no difference in immunity to lethal infection between sensitized and non-sensitized mice. Elimination of viable yeast cells from the infected foot was at the same rate as in non-sensitized mice. The cellular infiltrate was composed of predominantly PMN but included a significant mononuclear component.

Treatment of mice with CY resulted in a severe peripheral leukopenia, especially granulocytopenia. Resistance to lethal challenge was reduced significantly. Specific footpad responses in CY pretreated sensitized

mice were still seen indicating retention of immune reactivity. Ability to clear the localized infection was severely impaired.

In vitro experiments using murine PMN showed candidacidal activity. However, PMN from CY pretreated mice showed an altered ability to kill injested C. albicans. This defect could not be induced by prior incubation of normal mouse PMN in CY.

The importance of an intact non-specific host defense system has been shown by the results of this study. Immunological hypersensitivity may enhance the host response but have no effect on immunity. Among the non-specific host defense factors, a functional PMN seems to be of prime importance in resistance to infection by opportunistic organisms.

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