HOST RESPONSES TO ANTIGENS OF *CANDIDA ALBICANS*

by

SEYEDEH MOLOUK MOTAMEDI GETTNER

A thesis submitted for the degree of Doctor of Philosophy of the University of London

Mycological Reference Laboratory
London School of Hygiene and Tropical Medicine
Keppel Street
London WC1E 7HT

June 1979

ABSTRACT

Six different types of antigens were prepared from Candida albicans viz. whole cell homogenate (CAD-G), partially purified soluble mannan (M), particulate mannan adsorbed onto latex particles (M+L), purified cytoplasmic protein (PP), culture filtrate (CF) and killed whole cells (WC). The stimulatory effects of these antigens were tested on peripheral blood lymphocytes of 20 normal human subjects by in vitro lymphocyte transformation tests. The order of stimulation capacities was
CF > CAD-6 > WC > M+L > PP > M. Thus the crude antigens were found to be superior to purified and soluble preparations in promoting lymphocyte transformation.

The LD_{50} of test strain of C. albicans (NCPF 3153) to "TO" strain mice was estimated and calculated to be 1.52×10^6 blastospores. Seven groups of mice were immunized with the same antigens and challenged subsequently with a lethal dose of 4×10^6 live organisms. Live cells of C. albicans were also used to immunize mice before challenge. The agglutinin titres of the immunized animals ranged from 1:4 to 1:64. The group immunized with killed whole cells of Candida had highest agglutinin titres. The precipitin reactions were directed principally toward CAD-6 and purified cytoplasmic protein antigens. Different antigens conferred different degrees of protection in the following order:

CAD-6 > M+L > CF > PP > WC(A) = M > WC(K)

Heat killed vaccine (WC(K)) did not confer any measurable protection. Surviving animals showed agglutinin titres which ranged from 1:4 to 1:16 and precipitins which were directed primarily against

CAD-6 and PP antigens. None of the groups showed any anti-mannan precipitins.

Mice immunized with heat-killed whole cells of C. albicans and control animals showed the same responses to thigh muscle challenge with 5×10^8 live organisms. An abscess was formed which resolved spontaneously after 4 weeks with a cellular reaction mostly of polymorphonuclear cells. Candida was present mostly in the mycelial phase. Repeated thigh muscle challenge with live organisms produced local resistance manifested by accelerated sequestration of abscesses and the appearance of Candida mostly in yeast phase.

ACKNOWLEDGEMENTS

I am most of all grateful to my supervisor Professor D.W.R. Mackenzie for inspiration and his wise guidance all through this study. I am also thankful to Professor J.F. Soothill for his interest and generosity in making it possible for me to carry out part of this study at the Department of Immunology of the Institute of Child Health at Great Ormond Street. My gratitudes are due to Mrs Lorna Layward for her assistance and efficient teaching of the technique of lymphocyte transformation. My special thanks to Mr A.G. Proctor for his encyclopaedic knowledge and his willingness to share it at all times. My appreciation to all the staff of the Mycological Reference Laboratory particularly to Dr Veronica Hearn and Dr R.J. Hay who were always helpful with valuable suggestions. I am also indebted to Dr W.D. Brighton for providing the cytoplasmic protein antigen of the C. albicans, to Dr C.J.M. Rondle for the use of his photographic equipment and to all the subjects who gave their blood.

Finally I extend my appreciation to Mrs Miriam Brooks for the typing of the thesis.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (CONT)

INTRODUCTION

Candida albicans is a eukaryotic yeast-like fungus which may grow in the form of oval budding yeast cells (blastospores) measuring 2.5-8.0 μm in diameter, or as elongated cells joined end to end (pseudohyphae). Filamentation is often evident when the yeast is grown under reduced oxygen tension. At temperatures between 20-30°C and in nutritionally deficient media, thick-walled resting cells (chlamydospores) 7-17 μm in diameter may be formed. A distinct type of filamentation where sinuous elongated "germ tubes" are produced as outgrowths from budding cells is seen within 1-3 hr of incubation in mammalian serum at 37°C. This morphological conversion (dimorphism) is very distinctive and constitutes the basis of the germ tube identification test described by Taschdjian et al. (1960) and Mackenzie (1962).

Dimorphism can be induced in vitro as well as in the tissues of the host. The fungus occurs mainly in the form of budding yeasts as part of the normal flora. The transition from a saprophytic to an invasive state is often accompanied by a change from yeast to mycelial morphology. Simonetti and Strippoli (1973) reported that the yeast phase of C. albicans had a significantly higher pathogenicity than the mycelial form after intraperitoneal and intravenous injection in mice. However the mycelial form seemed to play an important role in the spreading of infection.

The dimorphism exhibited by C. albicans is not comparable to that seen with Histoplasma capsulatum, Coccidioides immitis and other causative agents of deep-seated mycoses, in the sense that

both yeast and filamentous forms are usually present in infected tissues. Although invasion of tissue may be by cells which are predominantly filamentous, it is erroneous to equate mycelial and blastosporic phases with invasive and saprophytic states respectively.

Principal factors regulating the proliferation of this fungus in man's body are glucose concentration, pH, availability of molecular iron and presence of other microorganisms in the normal flora. C. albicans is tolerant of acid conditions and favours a high concentration of glucose. It grows poorly in serum with unsaturated siderophilin (Caroline et al., 1964). Saturation of siderophilin with iron promotes growth of the fungus.

C. albicans has an affinity for the mucous membrane and the areas most frequently colonized are those surrounding body openings such as the oral cavity or vagina and the intestinal tract. The organism thrives on warm, moist surfaces. Man becomes colonized with Candida early in life and it becomes established within a few days of birth as a member of his normal flora. According to Winner (1969) 1% of newborn infants have Candida in their mouths, and by the end of the first year of life almost all children have been exposed to this organism. Marples (1966) stated that the yeast is present in the mouth of 40% of human adult subjects. Somerville (1964) found that vaginal carriage in pregnant women in a maternity hospital was 40% with a large decrease in the incidence postnatally to 9.6%. According to Morton and Rashid (1977) the frequency of isolation from any site will vary with climate, diet, age and health of the host as well as the diagnostic methods employed and the frequency of their use. He reported that positive findings in mouth and rectum varied between 6-54% and 17-75% respectively. Occurrence

of yeasts on healthy skin increased with age from 2% in adolescents to 27% in adults over 60 years. Somerville (1972) found that acquisition rate of C. albicans in a hospital for patients with skin diseases increased with age, being 28 and 37% respectively in the groups below and above 40 years of age.

Natural defence mechanisms of the host and their intactness are of critical importance in restricting the numbers of Candida cells and maintaining a state of commensalism. Lehrer and Cline (1969a) demonstrated that normal neutrophils and to a lesser extent eosinophils and monocytes have candidacidal activity. The major participants in Candida killing were found to be the lysosomal enzyme myeloperoxidase and its oxidant substrate hydrogen peroxide. Leukocytes of a patient with hereditary myeloperoxidase deficiency phagocytized C. albicans normally but failed to kill them. Yamamura and Valdimarrson (1977) reported that C. albicans opsonized by C3 deficient serum was ingested, but not killed in vitro by polymorphonuclear leukocytes. Killing could be induced by adding purified C3 to the deficient serum. It was concluded that C3 participates directly in the intracellular process leading to phagocytic killing of C. albicans. Morelli and Rosenberg (1971) also concluded that complement plays an important role in resistance to infection with C. albicans either by inactivating endotoxin of the organism or mobilizing phagocytic cells by generating chemotactic activity. Drew (1973) related chronic mucocutaneous candidiasis to abnormal function of serum complement. Three members of a family suffering from the disease had decreased activity of total serum complement.

Factors other than complement have been described in normal serum which affect host susceptibility to infection with Candida. Louria and Brayton (1964) described a "candidacidal" factor in normal human sera or plasma which was present in alpha and beta globulin fractions of the serum globulins but not in albumin or gammaglobulin. Chilgren et al. (1968) described a "clumping factor" of C. albicans present in normal sera which was inhibited by sera of patients with cutaneous candidiasis. Subsequently Louria et al. (1972) reported that 98% of adults between 21 and 50 years of age have clumping factor in their sera. This factor is absent in patients with systemic candidiasis. Demonstration that a serum lacks or interferes with clumping can be a good indicator of infection. Smith and Louria (1972) characterized the Candida clumping factor of normal rabbit serum as a macro-euglobluin of fast beta mobility and reported the gradual loss of the clumping activity during active immunization of rabbits with heat-killed Candida. The loss was clearly related to the appearance of humoral antibody rather than to a reduction in clumping factor. It appeared that antibodies to heat-killed Candida promoted mycelial transformation.

Exposure to this fungus or immunologically related organisms induces both humoral and cellular responses in man, shown by the development of specific serum agglutinins and manifestations of delayed (type IV) hypersensitivity. Drake (1954) tested 114 normal sera for agglutinins and found that 95% reacted with at least one of the 5 different species of yeasts used. C. albicans was agglutinated by 45% of the sera. Shannon et al. (1966) found that 85%

of the normal adult subjects demonstrated reactivity with Candida antigen. Cutaneous and lymphocyte reactivity to C. albicans is so widespread in the general population that Candida extracts are commonly used to detect abnormalities of the immune system.

The commensalism of C. albicans in its host is not always stable. Any of the factors which can alter the host defences and favour proliferation and establishment of the Candida can result in a change of its status from commensal to pathogen. C. albicans is therefore an opportunistic pathogen producing disease following local or generalized debilitation of its host. The organism is capable of causing various diseases in man ranging from superficial lesions, such as thrush, and allergic manifestation to more serious conditions such as mucocutaneous, chronic granulomatous and systemic candidiasis. Infancy, old age, pregnancy, endocrine disorders, malignant diseases, leukopaenia, trauma, post-operative state, antibiotics and immunosuppressive drugs are some of the known predisposing factors.

Experimental studies on modified animal hosts have confirmed some of the above predisposing factors. Salvin et al. (1965) reported that neonatally thymectomized mice had an increased susceptibility to C. albicans infection. Apparently contradictory findings were reported by Cutler (1976) who found that congenitally thymic-deficient (nude) mice were more resistant than phenotypically normal littermates to an intravenous injection of a lethal dose of viable C. albicans. Lack of susceptibility to experimental candidiasis in these animals was attributed to the existence and effectiveness of primary non-specific defence mechanisms such as phagocytosis.

Hurley (1966) reported that pre-existing infection of mice with Proteus morganii, total body x-irradiation of mice and alloxan-induced diabetes in rabbits made the animals more susceptible to fatal infection than the healthy controls. Mukherji and Basu Mallick (1972) found that mice challenged on the third day after treatment with the cytotoxic agent cyclophosphamide died within 48 hours while there was no mortality in control animals. Of interest was the finding that Candida was present in every organ examined in the mycelial form.

Experimental animals including rabbits, mice and guinea pigs are susceptible to infection with C. albicans although their susceptibilities vary markedly (Winner, 1956, 1960; Hurley and Fauci, 1975; Winblad, 1975). Intravenous injection of large numbers of live organisms is usually fatal. Rabbits die from uraemia: in guinea pigs death is due to acute pulmonary congestion and oedema. In all 3 types of experimental animal kidneys are the target organs with abscess formation in the cortex and cellular responses consisting of polymorphonuclear leukocytes, monocytes and lymphocytes. Louria et al. (1963) suggested that kidneys are more susceptible to invasion because the inflammatory response appears four hours later than in other tissues. Kernbaum (1975) concluded that the renal medulla is susceptible to C. albicans due to hyperosmolality and inactivation of the complement system. Hasenclever (1959) reported that Swiss, female mice are as or more susceptible to C. albicans than male albino rabbits. The disease is more acute in rabbits and death results after 1-7 days, while it is more chronic in mice and the animal may survive up to 30 days. Mouse models for experimental candidiasis have been described by Blyth (1958), Hurley and Winner (1963).

The mechanism of pathogenicity in man and animal is not known. Many investigators have suspected the presence of a toxin and tried to demonstrate its presence using techniques derived from studies on bacterial endotoxin. Salvin (1952) stated that dead cells of C. albicans have endotoxin-like properties. Winner (1956) suggested that an endotoxin may be responsible for the pathological effects produced by the organisms on infected tissues. Maibach and Kligman (1962) from an examination of experimental infections in the skin of human volunteers concluded that the pathological reaction in cutaneous candidiasis is mediated by endotoxin-like materials released by the organism. Isenberg et al. (1963) reported that a phenol extract of C. albicans contained an endotoxin-like substance. Dobias (1964) prepared an extract from yeast cells which contained sensitizing antigens and an endotoxin-like substance. Mankowski (1962, 1968) isolated a glycoprotein from culture filtrate which retarded the growth of new-born mice on repeated subcutaneous injections. Zaikina and Elinov (1968) described a plasmocoagulase in cell extracts of many species of fungi including Candida which caused enzymatical proteolysis of prothrombin with the formulation of thrombin or thrombin-like substances.

Chattaway et al. (1971) were unable to demonstrate toxin production by a pathogenic strain of C. albicans and concluded that toxin production appeared to be a strain characteristic and not a property of pathogenic strains per se. They suggested that toxic activity of the fungus may be related to local activity of enzymes elaborated by the organism on host tissue during its growth. Cutler et al. (1972) induced a granulomatous response in mice. He could

not however assign a role of toxicity to any identified components of the organism.

Iwata (1979) and his collaborators in Japan have made extensive studies on "canditoxin", a heat-labile protein with an LD_{50} for mice of 0.3 µg/gm body weight. Amongst the effects induced by administration of "canditoxin" to experimental animals were enhancement of infection with C. albicans, more marked histopathological changes and suppression of T-cell lymphocytes.

The immunological state of the host is a major factor in determining susceptibility of candidiasis. The literature contains many reports on the influence of cellular and humoral elements of the immune response on Candida infections of experimental animals and man. Data have been presented from many sources but there is not at present any uniformity of opinion on the protective roles of humoral and cellular responses respectively. Many of the reports have contradictory findings or conclusions. Comparisons of results from different studies are usually difficult or impossible to make because of the marked differences in experimental design and methods. Moreover earlier studies were made without the benefit of the clearer understanding of the basic nature, kinetics and significance of the immune response which has emerged only within the past decade.

Kurotchkin and Lim (1933) reported that passive immunization of rabbits subsequently challenged with C. albicans was not effective and could even have been deleterious. Hurd and Drake (1953) found that passive immunization of rabbits with sera containing agglutinin titres of 1:1280 to 1:2560 did not protect the animal

against challenge with lethal doses. They also concluded that active immunization not only failed to provide protection but was harmful to the experimental animals. Winner (1956, 1958) reported failure of active or passive immunization to protect rabbits against fatal infection with C. albicans. He concluded that antibodies induced by whole cell immunization were directed against surface components of the cell: unless the lethal mechanism of the yeast was associated with the cell surface there was no reason to suppose that the pathogenesis of infection would be influenced by these antibodies.

Experimental studies with mice have yielded some evidence to link humoral responses with enhanced protection to experimental infection. Mourad and Friedman (1961, 1968) reported that active and passive immunization of mice protected 50% of the vaccinated mice compared to controls. Dobias (1964) reported protection in mice following repeated subcutaneous injections of a cell wall preparation from C. albicans. Mazetti and Marraccini (1957) were successful in active immunization of guinea pigs. Formalinized-cell vaccinated animals survived while the untreated animals succumbed at 7 to 14 days post challenge.

The role of antibody in defence against C. albicans infection is widely regarded as minor compared to cell mediated immunity (CMI) (Kirkpatrick et al., 1971; Lehner et al., 1972; Butz-Jorgensen, 1973; Miyake et al., 1977).

The importance of CMI in resistance to Candida infection has been emphasized by studies demonstrating defects in delayed (type IV)

hypersensitivity or *in vitro* correlates of cellular immunity in patients with candidiasis. Buck and Hasenclever (1963) in a study of 297 women attending a prenatal clinic found that vulvovaginitis and growth of *C. albicans* occurred more frequently in women with negative than with positive skin tests. Absence or abnormality of type IV hypersensitivity have been reported from cases of chronic mucocutaneous candidiasis by Buckley *et al.* (1968), Landau (1968) and Kirkpatrick *et al.* (1971).

Chilgren *et al.* (1969) reported 3 patients with mucocutaneous candidiasis who had cutaneous anergy despite the fact that their lymphocytes had intact antigen recognition and processing mechanisms. The anergy seemed to result from a deficiency of mediator production, migration inhibition factor (MIF) or the presence of an inhibitor to this factor. Valdimarsson *et al.* (1973) found 4 main immunological patterns among 26 patients with chronic mucocutaneous candidiasis. One group had antigenic activation of lymphocytes *in vitro* but without the release of detectable MIF and failed to express delayed hypersensitivity. Another group failed to mount delayed hypersensitivity although their lymphocyte transformated and they produced MIF. A third group had a serum factor which selectively suppressed the response of lymphocytes to *Candida* antigen but these patients reacted to purified protein derivative (PPD) and could be sensitized to dinitrochlorobenzene (DNCB). The last group had no detectable abnormalities.

C. albicans rarely causes persistent infection in healthy subjects and the high incidence of positive skin tests to *Candida* suggests that specific acquired immunity is associated with a state

of commensalism. It may eventually prove to be true that enhanced resistance to Candida infection is related to a measurable extent to cellular immunity.

The reactions of lymphoid cell populations from sensitized subjects to fungal antigens has become a subject of interest. Such interactions result in a variety of biological activities, the nature of which depends on the environment in which the cellular response to antigen is taking place. Assessment of specific cell mediated immunity can be made by reference to four biological phenomena which are all mediated by lymphokines (Dumonde et al., 1969). These are:

1. Production of type IV (delayed hypersensitivity in vivo).
2. Transformation of sensitized lymphocytes in the presence of specific antigen in vitro.
3. Production of migration inhibition factor (MIF) following interaction of specific antigen with lymphoid cell population containing macrophages and sensitized lymphocytes in vitro.
4. The cytotoxic effect of sensitized lymphocytes on populations of "target" cells on whose surfaces the specific antigen(s) are borne in vitro.

In vitro studies by Alford (1973) showed that an antigenic extract prepared from C. albicans caused significant uptake of tritiated thymidine in lymphocyte cultures of normal adults compared

with hospital patients. Cells from all 25 normal subjects demonstrated lymphoblastic activity when exposed to Candida antigen. Precise information concerning the conditions required to induce cell mediated immunity would be helpful for prognosis and treatment of human diseases in which antibody does not play a role. The acquisition of specific cellular responsiveness depends on the route of stimulation of the host cells and the nature of the antigen and the manner of its presentation to the host.

Candida albicans has numerous antigenic components. Winner (1972) studying the ultrastructure of Candida has defined and mapped its major antigens. The cell wall has several zones. The external zone consists of mannan, protein and chitin. Internal to this there is a narrow, dark zone containing hydroglucans followed by a wider and lighter staining zone immediately adjacent to the cell membrane composed of glucans.

The three chemical categories of antigens of practical importance are mannan, usually complexed with protein, glycoproteins, and cytoplasmic protein. The cytoplasm is rich in enzymes and other macromolecules which are capable of acting as antigens. Axelsen (1973) using 2-dimensional immunoelectrophoresis has described 78 water-soluble antigens associated with the cell sap. It is not known which components are associated with tissue invasion, and although serological tests are widely used in the recognition and monitoring of patients with candidiasis, no ready and reliable distinction can be made between colonization and invasion. Antibodies are formed to many structural components and metabolites of C. albicans

and serological tests have received much attention in the past two decades. Correctly used and with a clear understanding of their limitations as well as their advantages they can provide valuable information to the clinician and they constitute an important diagnostic aid.

Three types of antigen are most commonly used in immunodiagnostic tests, viz. whole cell homogenates, intact non-viable organisms and culture filtrates. Disintegrated yeast cells contain a mixture of protein, glycoprotein, lipoprotein and polysaccharide antigens, derived from cell wall and cytoplasm. They are used in gel diffusion tests such as double diffusion or counterimmunoelectrophoresis in agar or agarose gel. Intact, non-viable cells are used for agglutination or indirect fluorescent antibody tests. Culture filtrate antigen is widely used for skin testing. Numerous modifications and variations have developed from these basic reagents and systems. The diagnostic laboratory now has a wide range of serological tests available for detection and measurement of antibodies, including agglutination of whole cell or inert particle such as latex agglutination or haemagglutination. Enzyme-linked immunosorbent assays and solid phase radioimmunoassays have also been evaluated. Complement-fixation tests are now rarely used. Antigens in common use vary greatly from laboratory to laboratory, and there are at present no internationally accepted reference reagents against which performances of serodiagnostic antigens and reference antisera can be compared. In addition to the principal categories of antigen used in immunodiagnostic tests (whole cell homogenates, intact cell, culture filtrates), attention has been paid increasingly to immunological

activities of polysaccharide (mannan) in contrast to cytoplasmic proteins. According to Northcote (1954) mannan accounts for 31% and glucan for 29% of the yeast cell wall. Mannan, unlike glucan, is soluble and it is produced in abundance from a Candida population whether it is being cultivated in vitro, or growing on mucosal surfaces in vivo. Antibodies which react to C. albicans mannan are almost always detectable in human serum provided a sufficiently sensitive test system is used (Bull and Mackenzie, 1979). They are the first antibodies to show a rise following proliferation or dissemination of Candida in vivo. Chew and Theus (1967) demonstrated precipitins to the mannan antigen in 48% of healthy adults and in 69% of patients with mucocutaneous candidiasis. Stanley et al. (1972) investigating precipitins to three antigens of C. albicans in the sera of pregnant women detected antibodies in 56 (18%) of the 303 women examined. They found a significant association between the presence of precipitating antibodies to C. albicans and its isolation from vagina and clinical evidence of mycotic vulvovaginitis. The most frequent response was to culture filtrate 13%, to mannan 5% and to cytoplasmic antigen 7%. In another study Stanley and Hurley (1974) found a higher incidence (47.5%) of precipitins to the three antigens of C. albicans in sera from 200 selected pregnant women with symptoms of vaginitis and with yeast cells in the vagina.

In recent years, reactivities to mannan and cytoplasmic protein have been evaluated separately and in the United Kingdom, national reference reagents have been produced for whole cell extract, purified cytoplasmic protein and mannan. These reagents are maintained

at the National Institute for Biological Standards and Control, Holly Hill, Hampstead, and made available for comparison with similar preparations prepared for investigative work at other centres.

Immune responses to different antigens of C. albicans are often dissimilar. Pepys et al. (1968) found that precipitins against C. albicans mannan were common in asthmatic subjects especially those with pulmonary eosinophilia. The frequencies observed were 22 of the 40 patients with asthma (55%) and 26 of the 36 patients with asthma and pulmonary eosinophilia (72.2%). Such precipitins were associated with a type III (Arthus) skin test reaction. Inhalation tests with culture filtrate containing both mannan and protein antigens, provoked systemic reactions 4 to 8 hours later and both immediate and late asthmatic reactions. Mannan antigens were found to be highly immunogenic and a single skin test resulted in the appearance of precipitins in all subjects. Longbottom et al. (1976) stated that purified protein of C. albicans (i.e. free of mannan) and purified cell wall mannan (free of protein) are both capable of eliciting type I (immediate) skin test reaction in man, and, following passive transfer of immune serum, in the monkey. These reactions to the different antigens were however mediated by different classes of antibody. Thus responses to the purified cytoplasmic protein were mediated by heat-labile, long-term sensitizing IgE antibody whereas the reactions to the mannan were mediated by heat stable short-term sensitizing antibody, presumed to be in the IgG class. This group of workers concluded that protein components were responsible for type IV (delayed reaction) but could also elicit type I and possibly type III reactions.

Whole cell homogenates of Candida stimulate most normal lymphocytes when cultured in vitro (Foroozanfar et al., 1974). The nature of the stimulating antigen(s) is (are) not known. Studies on purified Candida extracts (Report of the Subcommittee for Candida Standardization, of the International Union of Immunological Societies, 1975) have suggested that they do not stimulate lymphocytes in vitro. To date no satisfactory explanation has been advanced for this phenomenon. Indeed there is little evidence in the literature to confirm or refute the impression that with refinement of Candida antigens there is a progressive loss of reactivity demonstrable in vitro. In preparing and supplying Candida antigens for lymphocyte transformation studies during the past decade, Mackenzie (personal communication) has often been requested by investigators to avoid any purification procedures, since these preparations were recognizably less effective in testing cellular responses in vitro. The validity of this observation has not been subjected to a critical evaluation, but it can be considered feasible on the basis of existing immunological precepts. It is known that particulate antigens are more readily processed by macrophages than soluble products (Weir, 1973). This could explain why crude rather than purified antigens would be effective in vitro in tests for cell-mediated immunity.

In practical terms, however, crude antigens are less satisfactory than purified ones, since neither chemical characterization nor standardization can be readily obtained. There would be a clear advantage in using a simple rather than a complex antigen for CMI testing in vitro.

If the effectiveness of a Candida antigenic preparation is related to physical as well as chemical characteristics, then effectiveness and reproducibility in tests for CMI might be substantially improved by the combined use of defined antigens and inert particles of uniform size on which the antigens are adsorbed. The antigen chosen for evaluation of particulate versus soluble antigen was mannan. It was selected on the basis that its composition is more uniform than preparations of cytoplasmic proteins.

The aims of this study were to prepare different types of Candida antigens.

1. To test the stimulating capacity of the different antigens on peripheral lymphocytes of healthy human subjects.

2. To compare reactivities of soluble and insolubilized antigen. This would be achieved by making mannan particulate by attaching it to inert particles.

3. To study the cellular responses of immunized and control mice to thigh lesion challenge with live cells of Candida albicans.

4. To test the immunogenicity and protective value of different antigens of C. albicans in mice against lethal challenge with live organisms.

MATERIALS AND METHODS

TEST STRAIN OF CANDIDA ALBICANS

C. albicans strain NCPF 3153 was used throughout this investigation. It was maintained throughout the period of study by frequent subculturing on slopes of glucose-peptone agar. This isolate was originally deposited at the Mycological Reference Laboratory in 1965 by Dr. H. F. Hasenclever, National Institute of Health, Bethesda, Md., U.S.A. as a subculture of his strain A207. Since that time it has maintained its morphological and biochemical characteristics and its pathogenicity towards inbred mice: it is the standard strain of *C. albicans* used and distributed as such by the Mycological Reference Laboratory.

MEDIA

All chemicals listed were Reagent Grade.

Distilled water was obtained from a glass still.

1. Malt Extract Agar

Malt Extract (Boots)	2.0 gm
Agar (New Zealand)	1.5 gm
Distilled water to	100 ml

Autoclaved at 112°C for 15 min.

2. Glucose Peptone Agar

D Glucose Monohydrate	2.0 gm
Bacto-Peptone (Difco)	1.0 gm
Agar (New Zealand)	1.5 gm
Distilled water to	100 ml

Autoclaved at 112°C for 15 min.

3. Glucose Peptone Agar with Antibiotics

Ingredients as above with the addition of

Cycloheximide ("Acti-dion", Upjohn) (4 gm in 80 ml of acetone)	1.0	ml
Chloramphenicol (Parke-Davis) (0.5 gm in 100 ml of absolute ethanol)	1.0	ml

Autoclaved at 112°C for 20 min.

4. Glucose Peptone Broth

Bacto-Peptone (Difco)	10.0	gm
D glucose monohydrate	20.0	gm
Distilled water to	1	litre

Autoclaved at 112°C for 15 min.

5. Synthetic Medium of Lee (SML)*

Ammonium sulphate	5.0	gm
Magnesium sulphate (hydrated)	0.2	gm
Dipotassium hydrogen phosphate (anhydrous)	2.5	gm
D-Glucose	12.0	gm
L-Amino acids		
Alanine	1.5	gm
Leucine	1.3	gm
Lysine	1.0	gm
Methionine	0.1	gm
Ornithine	0.0714	gm
Phenylalanine	0.5	gm
Proline	0.5	gm
Threonine	0.5	gm
Biotin (added after autoclaving)	0.001	gm
Distilled water to	1	litre

Autoclaved at 112°C for 20 min.

* Lee, et al. (1975)

6. Tissue Culture Medium RPMI 1640 (Difco) to which the Following were Added

2.4% 1 M Hepes (N-2-Hydroxyethylpiperazine-N-2-ethane-sulphonic acid (Hopkin and Williams))

1 mg/ml "ampiclox" (Beecham)

0.5 M NaOH to pH 7.4

Sterilized by filtration through 0.22 um millipore membrane ("Millipore")

BUFFERS AND SOLUTIONS

1. Volatile Buffer pH 7.3

0.4% ammonium hydrogen carbonate

2. Borate Buffer (pH 8.2) for Double Diffusion

Boric acid	10.0 gm
Borax	20.0 gm
Ethylene diaminetetra-acetic acid disodium salt (EDTA)	5.0 gm
Distilled water to	1 litre

This buffer is double strength and it is mixed with an equal volume of 1% agar in distilled water.

3. Buffered Agar for Double Diffusion

Oxoid agar No. 1	1.0 gm
Distilled water to	50.0 ml

Melt the agar and add 50 ml of warm borate buffer.

4. Veronal Buffer (Barbitone) pH 8.2

Barbitone	3.44 gm
Sodium barbitone	7.57 gm
Sodium azide	0.5 gm
Distilled water to	1 litre

5. Buffered Agar for Counterimmunoelectrophoresis pH 8.2

Purified agar (Oxoid)	0.5 gm
Agarose (indubiose A 37), IBF	0.5 gm
Veronal buffer to	100.0 ml

6. Saline Phosphate Diluent (for Agglutination Test) pH 7.2

Sodium chloride	8.0 gm
Sodium dihydrogen phosphate	0.8 gm
Sodium azide	0.65 gm
Distilled water to	1 litre

7. Citrate Buffer pH 7

0.1 M Citric acid (21.0 gm/l water)	6.5 ml
0.2 M Disodium phosphate (35.6 gm/l water)	43.6 ml

8. Phosphate Buffer Saline (Dulbecco A PBS) pH 7.3

Sodium chloride	8.0 gm
Potassium chloride	0.2 gm
Disodium hydrogen phosphate	0.15 gm
Potassium dihydrogen phosphate	0.2 gm
Distilled water to	1 litre

9. Glycine Saline Buffer pH 8.2

Glycine	14.0 gm
Sodium hydroxide	0.7 gm
Sodium chloride	17.0 gm
Sodium azide	1.0 gm
Distilled water to	1 litre

10. Buffer for Giemsa Stain pH 7.3

Disodium hydrogen phosphate (anhydrous)	5.0 gm
Potassium dihydrogen phosphate	2.1 gm
Distilled water to	1 litre

11. Washing Solution for gel precipitin tests

Sodium chloride	4.0 gm
Borax	4.0 gm
Distilled water to	1 litre

12. Antigen Diluent

Veronal buffer to which 0.02% merthiolate is added.

13. Antisera Diluent

1/5000 merthiolate in distilled water.

14. Glutaraldehyde Solution

Glutaraldehyde (2% aquous solution)	20.0 ml
Sodium bicarbonate	3.0 gm
Distilled water to	1 litre

15. Fehling's Solution

Solution A

Copper sulphate	3.5 gm
Rochelle Salt (potassium sodium tartrate)	1.7 gm
Distilled water to	100.0 ml

Solution B

5% Sodium hydroxide

Working solution

40 ml of solution A is mixed with 40 ml solution B.

16. Ficoll-Triosil

Ficoll 400 (Pharmacia Fine Chemicals)	90.0 gm
Distilled water	1227.0 ml
Triosil 440 (Nyegaard & Co., Oslo)	189.0 ml

17. Scintillation fluid

Toluene	2.5 litre
PPO (2.5 diphenyloxazole) (Fisons)	10.0 gm
POPOP (1,4-Di-2-(4-methyl-5-phenyl-oxazolyl benzene) (BDH)	0.25 gm

PREPARATION OF ANTIGENS

1. Whole Cell Homogenate (CAD-6)

This antigen was prepared from blastospores of C. albicans (NCPF 3153) grown for 3 days in stirred glucose-peptone broth culture (p. 22). After harvesting and washing, it was frozen at -20°C for 24 hr. Cells were then suspended in volatile buffer (p. 23) at a concentration of 20% (w/v), then processed in a "Dyno-mill" (Glen Creston) cell disintegrator which resulted in complete disruption of both cell wall and cell contents. The crude suspension was

15. Fehling's Solution

Solution A

Copper sulphate	3.5	gm
Rochelle Salt (potassium sodium tartrate)	1.7	gm
Distilled water to	100.0	ml

Solution B

5% Sodium hydroxide

Working solution

40 ml of solution A is mixed with 40 ml solution B.

16. Ficoll-Triosil

Ficoll 400 (Pharmacia Fine Chemicals)	90.0	gm
Distilled water	1227.0	ml
Triosil 440 (Nyegaard & Co., Oslo)	189.0	ml

17. Scintillation fluid

Toluene	2.5	litre
PPO (2.5 diphenyloxazole) (Fisons)	10.0	gm
POPOP (1,4-Di-2-(4-methyl-5-phenyl-oxazolyl benzene) (BDH)	0.25	gm

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centrifuged at 10,000 g for 1 hr and the supernatant concentrated by dialysis against 10% polyethylene glycol at 4°C, to 20% of its initial volume. The concentrated material was centrifuged again at 10,000 g for 1 hr and the supernatant lyophilized.

This antigen is used routinely for detection of antibodies to C. albicans in serum submitted to the Mycological Reference Laboratory for serodiagnostic studies.

2. Mannan (M)

Mannan was prepared according to the method described by Peat et al. (1961) as modified by A.G.J. Proctor (personal communication). C. albicans was grown in 300 ml of glucose broth for 2½ days with magnetic stirring and then transferred to 6 two-litre ribbed conical flasks each containing 1000 ml of SML medium (p. 22). The flasks were incubated at 35°C on a rotary incubator (Gallenkamp) at 110 rpm for 60 hr. Candida cells were harvested by means of a continuous rotor centrifuge (MSE High Speed 18) at 21,000 g with flow rate of 250 ml/min. The yield was 105 gm. Harvested cells were collected in a 250 ml polypropylene beaker, frozen at -40°C, and then thawed. After the addition of 200 ml of citrate buffer at pH 7 (p. 23) they were distributed in two 250 ml polypropylene centrifuge bottles and autoclaved for 2 hr at 126°C (20 lb pressure). The cream-coloured suspension obtained was centrifuged at 400 g for 30 min. The supernatant, a yellowish liquid, was collected and retained. Sediment from the two bottles was resuspended in 200 ml of water, centrifuged as above and the resultant supernatants added to the previous one. To the combined supernatants 55 ml of glacial

acetic acid (99.6%) was added slowly from a graduated cylinder and mixed until a precipitate formed. After centrifugation for 30 min at 4000 g, the supernatant was neutralized to pH 7 with N/10 sodium hydroxide and its volume measured in a 500 ml graduated cylinder. Two volumes, (1000 ml) of cold ethanol were placed in a 2 l flask and the supernatant was added to it while stirring. It was then covered with aluminium foil and stored at $4^{o}C$ overnight.

The clear supernatant was aspirated by vacuum pump and the loose residue centrifuged in a polypropylene tube. The resultant gum-like sediment was washed with 50 ml of 60% ethanol, centrifuged, and the excess liquid drained off on a filter paper. The solid residue was dissolved in 50 ml of water and 80 ml of freshly made Fehling's solution (p. 25) was added slowly until a precipitate was formed. This was centrifuged and 50 ml of water was added to the sediment. Concentrated hydrochloric acid was added drop by drop until the copper complex dissolved. The volume was measured (54 ml) and three volumes (162 ml) of ethanol was added to the solution while stirring. It was kept at $4^{o}C$ overnight and then centrifuged at 4000 g for 30 min. The sediment was dissolved in 50 ml of water and re-precipitated with 150 ml of ethanol which was added gradually while stirring. It was placed at $4^{o}C$ and the above steps repeated. The precipitate was finally dissolved in 10 ml of water and kept at $4^{o}C$ until freeze-dried in an Edwards EFO 3 freeze drier. A total yield of 400 mg of mannan was obtained in the form of discrete white flakes. This was tested for antigenic activity against rabbit serum containing antibody to mannan by counterimmunoelectrophoresis (p. 38). One or two distinct precipitin lines were obtained depending on the concentration of antigen used.

3. Particulate Mannan (M+L)

The initial attempt to render mannan insoluble was made with "Sigma cell". With this procedure, 5 gm of cyanogen bromide were dissolved in 100 ml of distilled water and mixed with 5 gm of "Sigma cell" (50 μm cellulose particles, Sigma Chemical Co.) suspended in 100 ml of distilled water. The pH of the suspension was maintained between 10 and 11 for 5 min. with 1 M sodium hydroxide which was added dropwise. After washing with 5 l of ice cold 0.1 M sodium bicarbonate using a Buchner funnel, the Sigma cell was washed with coupling buffer (76.8 ml of 1 M sodium bicarbonate and 7.74 ml of 1 M sodium carbonate made up to 1 litre with distilled water).

Twenty-five mg of antigen was dissolved in 0.7 ml of coupling buffer, mixed with the activated Sigma cell and left overnight at 4°C. The Sigma cell was washed 3 times with 0.5 M sodium bicarbonate, followed by ethanolamine buffer (6.2 ml ethanolamine/1 of coupling buffer) and a third wash overnight. Finally it was washed twice with 0.2 M glycine hydrochloric acid buffer pH 2.8 (0.2 M glycine and 0.2 M hydrochloric acid).

Sensitization of latex

The stock suspension of latex particles (Difco, 0.81 μm) was diluted 1:10 with glycine-saline buffer (p. 24). Twenty mg of mannan was added to each ml of glycine-saline buffer. An equal volume of diluted latex suspension was added to the antigen mixture, mixed well and left at 4°C for 24 hours. The sensitized latex particles were washed twice with glycine-saline buffer by centrifugation at

18,000 g for 30 mins. The original volume was restored with glycine-saline buffer and the particles were stored at 4°C until required.

The preparation was tested for antigenicity by setting up an agglutination test (p. 38) with rabbit serum containing antibody towards mannan. The amount of mannan left in the supernatant was determined by the method of Dubois et al. (1956). It was calculated that approximately 25% (2.5 mg) of the mannan present in the original suspension was bound to 1 ml of latex particles.

4. Purified Cytoplasmic Protein (PP)

This antigen was obtained from the National Institute for Biological Standards and Control, Holly Hill, Hampstead, London. It was prepared by rupturing yeast phase of C. albicans (NCPF 3153) and purifying the protein antigens by affinity chromatography using a column containing Sepharose 4 B conjugated with concanavalin A. Passage through the column removed all traces of mannan. (Brighton W., personal communication).

5. Culture Filtrate (CF)

C. albicans was grown in 400 ml of SML medium (p. 22) for 7 days at 25°C on a rotary incubator at 110 rpm. After centrifugation the supernatant was dialyzed against 30% polyethylene glycol at 4°C overnight to an end point of 2% of its original volume. It was freeze-dried in an Edward's High Vacuum EFO 3 apparatus and stored in vacuo. Its antigenic activity was tested with rabbit and sheep serum containing antibodies for C. albicans by double diffusion tests (p. 40).

Whole Cell Killed (WC)

C. albicans was grown on 5 slants of 2% malt extract agar (p. 21) incubated for 24 hr. at 28°C. The growth was harvested and washed 3 times with sterile distilled water. Non-budding organisms of uniform size were selected by use of a sucrose density gradient. Sucrose solutions of 20%, 30% and 40% in distilled water using Analar sucrose were prepared. In matched 50 ml round bottom glass centrifuge tubes 8 ml quantities of the different sucrose solutions were carefully layered one above the other, with a 10 ml syringe, in the order 40%, 30% and 20%. Three ml of the C. albicans suspension was layered on top of the lightest solution and centrifuged at 160 g for 5 min. The cells of uniform size were collected from the top of the 30% sucrose with a Pasteur pipette and examined microscopically and found to consist of more than 95% of unbudding yeast forms. The cells were adjusted to a concentration of 10^6/ml by the aid of a haemacytometer (Improved Neubauer) and killed by heating at 70°C for 1 hr. The viability of the organism was tested by methylene blue technique of Lehrer and Cline (1969b) and also by culture on glucose peptone agar.

HUMAN SUBJECTS FOR STUDIES OF LYMPHOCYTE RESPONSES

Twenty healthy human volunteers including members of laboratory staff, students and visitors were tested for their cellular responses to the 6 different preparations of Candida albicans. They consisted of 8 females whose age ranged from 25 to 65 years with a mean average of 45 years and 12 males with the age range between 20 to 55 years with a mean average of 40. They were bled by venepuncture from antecubital vein in the forearm and their lymphocyte responses were determined by transformation studies in vitro (p. 31). Not all subjects were tested with all the test antigens (Table 2).

Serum antibody titres for C. albicans were determined by whole cell agglutination (p. 38), particulate mannan agglutination and counterimmunoelectrophoresis (p. 38).

LYMPHOCYTE TRANSFORMATION

Preparation of Lymphocytes

Ten to 15 ml of venous blood was defibrinated in a sterile 20 ml universal tube (Sterilin) containing 10 glass beads. After centrifugation for 10 min. at 200 g the plasma was collected into a 5 ml universal tube with a sterile Pasteur pipette. The cells were resuspended with sterile Dulbecco phosphate buffer saline (p. 24) and overlaid gently on 10 ml of Ficoll-Triosil (p. 25) and centrifuged for 30 min at 400 g. After centrifugation erythrocytes and granulocytes were present in the sediment and lymphocytes at the interphase. The interphase was transferred by sterile Pasteur pipette into universal tube and washed 3 times for 5 min. each in phosphate buffer saline centrifuged at 200 g. The cells were resuspended at the end of each wash using a Rotomixer (Fisons) and finally resuspended in 5 ml of RPMI 1640 culture medium (p. 22) and counted in a haemocytometer. The cells were adjusted to 10^6/ml with culture medium containing 10% homologous plasma.

Lymphocyte Cell Culture

Cultures were set up in duplicate for each of the test antigens in a sterile round bottomed microtitre plate (Linbro). The concentrations of antigens used for soluble antigens were 2000, 200, 20 and 2 μg/ml. Particulate mannan (M+L) was used in

concentrations of 2500, 250, 25, 2.5 μg/ml. The whole cell killed antigen (WC) was used in concentrations of 10^6, 10^5, 10^4, 10^3/ml (Table 3). Two hundred μl of lymphocytes and 10 μl of each antigen concentration were added to each well except the control which did not receive antigen. The plates were sealed with a plate sealer (Flow Laboratories) and incubated at 37°C for 5 days.

Harvesting and counting

Ten μl of tritiated thymidine (0.2/μCi, Radiochemical Centre) was added to each well with a Hamilton syringe for the last 24 hr of culture. The plate was resealed and the cells resuspended. The cultures were harvested on a cell harvester (designed by Department of Immunology at the Institute of Child Health, Great Ormond Street, London) and collected onto Whatman CF/C filters 2.5 cm. The filters were placed in plastic scintillation vials (Packard) and left at room temperature to dry. Six ml of scintillation fluid (p. 25) was added to each vial which were then capped. Each vial was counted for 4 min in a beta counter (1210 ultrobeta-LKB). A background count and a hexadecane standard (Radiochemical Centre) was added to each run and results expressed as dpm corrected for the efficiency of the counter and the background.

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DETERMINATION OF LD_{50} IN "TO" MICE

Pathogenicity of strain NCPF 3153 of C. albicans used in this study was tested by determination of LD_{50} value in experimentally infected mice. Outbred mice (TO strain) 6-7 weeks old, with an average weight of 20 gm were used throughout. They were supplied with R+M No. 1 expanded maintenance diet (B.P.) and water ad libitum.

Growth from slants of 2% malt extract agar (p. 21) incubated at 28°C for 48 hr. was washed 3 times with sterile distilled water at 15,000 g for 5 min. and resuspended in sterile saline. Cell numbers were adjusted to 5×10^7/ml with the aid of a haemocytometer and confirmed by plate dilution counts with glucose peptone agar (p. 21). Seven groups of mice (5 or 10 animals per group) 6 weeks old (average weight 18 gm) were inoculated intravenously with graded doses of the washed suspension of C. albicans in 200 µl quantities. Doses used were: 5×10^4, 10^5, 5×10^5, 10^6, 5×10^6, 10^7, and 5×10^7. Observations were made daily for 30 days. Results showing cumulative deaths are shown in Table 6. LD_{50} was determined by the method described by Reed and Muench (1938).

Studies for the presence of C. albicans in different organs of mice dying after challenge with 5×10^6 cells were made on 2 mice. Kidneys, brains, lungs and livers were taken out of dead mice. An impression smear was made from each organ and stained by Giemsa (p. 43). About 1 gm of each organ was weighed in a sterile 30 ml masticator bottle and sterile saline in a concentration of 1:10 w/v was added. The tissues were macerated with a tissue

top drive homogenizer (MSE) and different dilutions made with sterile saline viz. 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-7}. Each dilution was cultured on a glucose peptone agar plate containing antibiotics (p. 22). Plates were incubated for 48 hr. and the number of colonies for each dilution obtained and averaged.

Portions of the above organs were fixed in 10% formal-saline for 48 hr. and transferred to 70% alcohol, dehydrated through increasing concentration of alcohol and toluene and embedded in paraffin (p. 41). Five μm thick histological sections were cut and stained with haemotoxylin-eosin (p. 41) and periodic acid-Schiff method (p. 42).

THIGH MUSCLE CHALLENGE IN MICE

The thigh lesion technique described by Selbie and O'Grady (1954) and developed by Pearsall and Langunoff (1974) was chosen in order to study the cellular responses during the course of experimental infection with C. albicans. It was anticipated that animals immunized with C. albicans would show different cellular infiltration of the thigh lesion compared to the control animals.

Heat killed vaccine was prepared by growing C. albicans on 2% malt extract agar slants at 28°C for 24 hr. The organisms were harvested and washed 3 times with sterile distilled water and resuspended in saline. The number of cells was adjusted to 5×10^8 by the aid of haemocytometer and confirmed by counts in glucose agar plate (p. 21). They were killed by heating at 70°C for 1 hr. Their viability was tested by the methylene blue method described

by Lehrer and Cline (1969b) and also by culturing on slants of glucose agar.

Three groups of TO mice were used for this study with 5 animals in each group. One group was immunized subcutaneously (s/c) at the nape of the neck with 0.25 ml of 5×10^8 of heat killed organisms mixed with an equal volume of complete Freund's adjuvant. Another group was injected intraperitoneally (i/p) with the same dose. The injections were repeated in both groups two weeks later. The animals were bled from a tail vein and their humoral responses tested by whole cell agglutination, double diffusion and counterimmunoelectrophoresis (p. 38). The immunization schedule was continued in both groups of animals with the same dose but without adjuvant at biweekly intervals for 2 months. The adjuvant was eliminated in later injections to minimize tissue irritation.

Immunized and control animals were challenged with live organisms by the injection of 5×10^8 cells in 0.2 ml quantities into the right thigh muscle. Challenge inoculum was prepared as described for the immunization inoculum, except that the cells were not killed. After preparation, they were maintained at 4^oC and used within 24 hr.

Ten days after the challenge when the infection was at its height one animal from each group was killed and the thigh lesion removed. Direct smears were made from the lesion and stained by Giemsa (p. 43) and PAS procedures (p. 42). The dissected lesion was then fixed in 10% formal saline and processed for histological

examination. At autopsy it was noticed that the spleens of the test animals were enlarged compared to the controls (Fig. 13). They were removed, weighed and processed for histological sections.

Thigh lesions in the remaining animals were self limited, disappearing after one month. They were reinfected in the opposite thigh muscle and the above challenge procedure was repeated. The last set of mice were reinfected for the third time in the right thigh muscle and on the fifth day of infection the lesions were removed and processed for histological sections.

PROTECTION STUDIES BY DIFFERENT ANTIGENS OF C. ALBICANS

Eight groups of TO mice, 6 weeks old, 10 animals in each group, and weighing an average of 20 gm were used in this study. Seven groups were immunized with different antigen preparations of C. albicans which consisted of 4 soluble and 3 particulate antigens. The soluble antigens were CAD-6, M, PP, and CF and the particulate antigens were heat killed Candida (WCK), and live Candida cells (WCA) and M+L. These antigens were prepared exactly as described for the in vitro studies.

Soluble antigens were given s/c in 6 doses over a period of 3 weeks. Each mouse received a total of 10 mg of the antigen. The particulate antigens were given i/p except for the live organisms which were given s/c in 3 doses over a period of 3 weeks. A total of 10^8 of dead organisms and 2×10^7 cells of live organisms and 1 ml of mannan on latex containing 2.5 mg of mannan per ml were used for each group concerned.

Seven days after the last immunization, the animals were bled individually from the tail. Sera from each group were pooled and tested for agglutinins by whole cell agglutination and for precipitins by counterimmunoelectrophoresis (p. 38). The animals were then challenged with 4×10^6 live cells i/v in the lateral vein of the tail and observed for 31 days. Deaths were recorded each day. The surviving animals were bled from the heart and then killed. All animals were autopsied. Spleens were removed and weighed. The average weight for each group was determined. Their pooled sera were tested for agglutinins to whole cells and for precipitins directed to 3 antigens viz. CAD-6, mannan and cytoplasmic protein.

Additional precipitation tests were done with mannan antigen treated with pronase to destroy traces of protein and the purified protein antigen treated with sodium periodate to oxidize traces of mannan using the method described by Hollingdale (1974). Pronase treatment of mannan consisted of adding 0.1 ml of pronase (Koch-light) 10 mg/ml in saline to 0.5 ml of mannan (5 mg/ml) and incubating at 37°C for 6 hours. A second volume of 0.1 ml of pronase was added and incubated at 37°C for 18 additional hours. As a control, antigen was treated with PBS instead of pronase.

Sodium periodate treatment of purified protein consisted of adding 0.1 ml of 0.02 M sodium periodate (Hopkin and Williams) which amounted to 42 mg/ml in water to 0.5 ml of purified protein (5 mg/ml) and incubated at room temperature (21°C) in the dark for 24 hr. As a control, antigen was treated with PBS instead of sodium periodate.

A comparison of the individual survival times of the mice in the experimental groups with those in the control group was made using the Mann-Whitney U ranking test (Von Fraunhofer and Murray, 1976). Fisher's exact probability test was used to compare the numbers of surviving and dead animals of each experimental group vs. the control group (Swinscow, 1976).

SEROLOGICAL TECHNIQUES

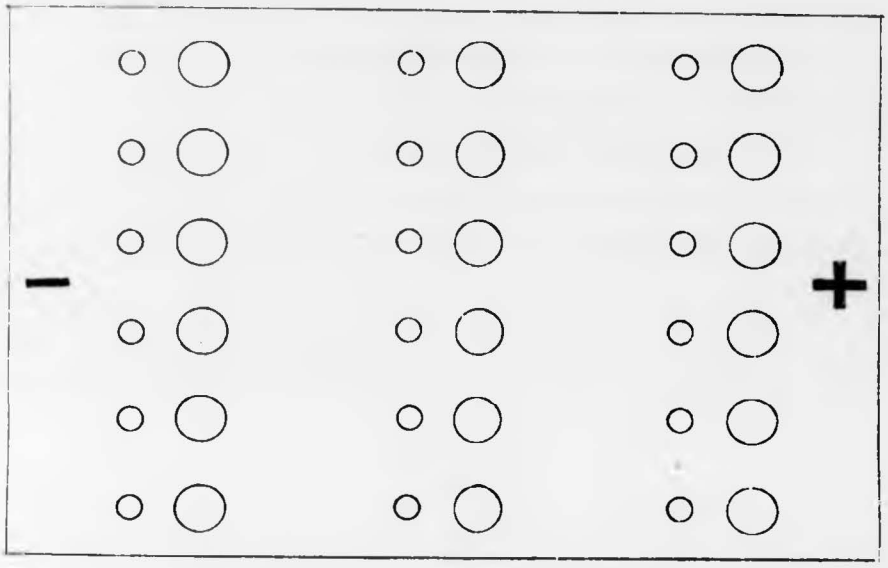
Agglutination

Serial two-fold dilutions of the test sera were made in phosphate buffer pH 7.2 (p. 23) in rigid plastic serological trays (WHO pattern). Phosphate buffer in 0.4 ml quantities was dispensed in each well of the row except for the first well which received 0.6 ml. Serial dilutions were prepared by adding 0.2 ml of the test sera to the first well (1:4), mixing and transferring 0.4 ml in turn to other wells in the series. 0.4 ml of the final well was discarded. One drop (0.02 ml) of 2% of *C. albicans* heat killed suspension was added to each well. After thorough mixing, the plate was covered with a fitted plastic cover and left at room temperature for 24 hr. The sediment of each well was then resuspended by gentle shaking and the end point was read against dark background illumination.

Counterimmunoelectrophoresis (CIE)

According to the method described by Mackenzie and Philpot (1975) 5.5 ml of melted barbitone buffered agar:agarose gel (p. 23) was layered on a 3" x 2" (75 mm x 50 mm) glass microscope slide and allowed to set, giving a gel 1.4 mm thick. Wells were prepared using stainless cutters

of appropriate size and a plastic template that fits closely over the slide without touching the gel surface (see pattern below).

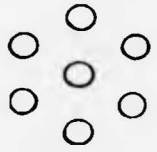


The diameter of serum wells was 4.5 mm and that of the antigen wells 2.5 mm, with edge-to-edge distances of 3 mm between the wells. Ten μl of antigen and 20 μl of serum was added to the appropriate wells and the slide was placed in an electrophoresis apparatus (Shandon) containing barbitone buffer (p. 23). A current of 4 v/cm was applied through absorbent tint wicks for 90 min. The slide was immersed in washing solution (p. 24) for 24 hr., followed by 10 min. in gluteraldehyde solution (p. 24), 10 min. in distilled water and 10 min. in tap water and finally dried in a hot air cabinet

at 45°C for 2 hr. The slide was then stained by Coomassie Brilliant Blue R (p. 44) to show specific antigen:antibody precipitins.

Double Diffusion Test (DD)

Three ml of melted buffered agar (p. 23) was placed in a 5 cm plastic petri dish. When set the arrangement of wells shown in the figure below was obtained by means of a pattern cutter. This was constructed from perspex and stainless steel. Individual wells were 4 mm diameter, with edge-to-edge distances of 5 mm.



Excess agar was removed from the wells by aspiration. Test serum was generally placed in the central well and antigens in peripheral wells. At other times antigen was placed in the central wells and test sera in the peripheral wells. Diffusion was allowed to proceed for 3 days at room temperature in a moist chamber. Plates were washed in washing solution (p. 24) for two days, then rinsed in distilled water for 10 min. The agar containing the well pattern was removed with a scalpel, placed on a microscope slide and dried at room temperature overnight. Dried slides were stained with Coomassie Brilliant Blue R.

TISSUE PROCESSING AND STAINING

Tissues were fixed in 10% formal saline for 48 hr. then placed in 70% alcohol for 24-48 hr. They were then transferred to a Shandon Elliott Duplex Processer and passed through the following solutions for two hours each.

80% alcohol
95% alcohol (twice)
100% alcohol (three times)
Toluene (twice)
Wax (Polywax-Difco) (twice)

They were embedded in wax and cut in 5 μm sections.

Haemotoxylin and Eosin Staining

Solutions

Cole haemotoxylin (Cole, 1943)

Haemotoxylin	1.0	gm
Warm water	200	ml
5% potassium permanganate	2	ml
Saturated ammonium alum	800	ml

Eosin

1% aqueous solution

Acid alcohol

1% hydrochloric acid in methanol

Procedure

Slides were carried through:

Xylene	15 min.
Absolute alcohol	5 min.
90% alcohol	5 min.
70% alcohol	5 min.
Water	5 min.
Haemotoxylin	20 min.
Tap water	rinsed
Acid alcohol	rinsed
Water	rinsed
Eosin	1 min.
Water	rinsed
70% alcohol	1 min.
90% alcohol	1 min.
Absolute alcohol (twice)	1 min.
Xylene (twice)	5 min.

Periodic Acid-Schiff Method of Staining (PAS)

Solutions

Periodic acid

1% aqueous

Potassium metabisulphite

1% aqueous solution to which a few drops of concentrated hydrochloric acid was added.

Schiff Reagent

Basic Fuchsin	1 gm
Water (90-95^{o}C)	100 ml

filtered at 50-60^{o}C and cooled

Sodium bisulphite	2 gm
N hydrochloric acid	20 ml

stoppered tightly and stored overnight in dark at room temperature — 300 mgm

Finely powdered charcoal was added and the suspension shaken for 1 min., then filtered through Whatman No. 1 filter paper.

The resulting light yellow solution was stored at 5^{o}C and discarded when any pink tint appeared.

Light Green (Raymond A. Lamb)

1% aqueous

Procedure

Slides were carried through:

Xylene	15 min.
Absolute alcohol	5 min.
90% alcohol	5 min.
70% alcohol	5 min.
Water	5 min.
Periodic acid (1%)	30 sec.
Potassium metabisulphite (twice)	10 min.
Running tap water	3 min.
Light Green (1%)	1 min.
70% alcohol	1 min.
90% alcohol	1 min.
Absolute alcohol	5 min.
Xylene	

The slides were mounted with DPX (BDH).

Giemsa Staining

Working Solution

Giemsa stock solution (Hopkin and Williams)	1 ml.
Buffer for Giemsa (p. 24)	2 ml.
Distilled water	8 ml.

Procedure

Slides were fixed in methyl alcohol and stained with a working solution of Giemsa for 30 min. The slides were then washed in running tap water for 3 sec.

Coomassie Brilliant Blue R Staining

Solutions

Coomassie Brilliant Blue R (R.A. Lamb)	0.5 gm
Diluent	100 ml

Diluent

Ethanol	450 ml
Glacial acetic acid	100 ml
Distilled water	450 ml

Procedure

Slides were stained with Coomassie Brilliant Blue R for 10 min. and then rinsed in methylated spirit and differentiated twice in diluent for a total of 3 min. to clear the background.

RESULTS

TRANSFORMATION OF HUMAN LYMPHOCYTES BY DIFFERENT ANTIGENS OF CANDIDA ALBICANS

The lymphocytes of most subjects responded to C. albicans antigens (Table 1). In comparing the 6 antigenic preparations, culture filtrate (CF), whole cell homogenate (CAD-6) and particulate mannan (M+L) were superior to purified protein (PP) and soluble mannan (M). The order of stimulation capacity was in the order: CF > CAD-6 > WC > M+L > PP > M (Fig. 1, Table 2). Thus the particulate and crude antigens used were found to be superior to purified and soluble preparations in promoting lymphocyte transformation.

Mannan adsorbed onto "Sigma cells" was not a satisfactory antigen in the lymphocyte transformation test since the particles would not stay in suspension and sedimented too quickly. Latex particles proved to be much more suitable as the particulate carrier for mannan and stimulated the lymphocytes of most of the subjects tested. This was in contrast to soluble mannan. The percentage of reactors to soluble mannan and mannan adsorbed onto latex was 5.2% and 82.4% respectively. To eliminate the possibility of nonspecific stimulation produced by latex alone, lymphocyte cultures were exposed repeatedly to untreated latex particles but without ever causing stimulation.

In the test system used, background levels of disintegration per minute (DPM) in the absence of test antigen varied from subject to subject (Table 1). The values obtained ranged from 802 (Subject EM) to 9065 (Subject SG). Control counts were obtained

for the cells of each subject each time a different antigen was tested. The range of the figures obtained for control counts for each subject was usually a narrow one. Thus, for 14 of the 20 subjects, the arbitrary ratio $\frac{\text{High count - low count}}{\text{average count}}$ was 0.5. In 5 subjects (MP, LG, FD, RT, JK) the control values had a somewhat broader range, with arbitrary ratios from 0.45-0.83 respectively, and in one volunteer (SG) the control counts varied from 1342-9065 DPM with an arbitrary ratio of 1.48. Because of this variable range, control values were not estimated by determination of average. Instead, the control value was taken as the figure obtained for each series of antigen dilution tested, when antigen was omitted from the lymphocyte tissue culture medium.

In comparing the potency of the antigens CF, CAD-6 and M+L were the most potent of the antigens tested and even at levels of 0.1 μg/ml stimulated the lymphocytes of some subjects. Purified protein and soluble mannan and whole cell were the least potent and were not able to stimulate lymphocytes when used at concentrations below 10 μg/ml (Table 3). Comparison of degrees of stimulation indices of lymphocytes from each of the 20 volunteers to 6 test antigens are indicated in Table 4 and Fig. 2.

TABLE 1 STIMULATION OF LYMPHOCYTES IN 20 SUBJECTS TO 6 ANTIGENS OF C. ALBICANS EXPRESSED AS DPM (DISINTEGRATION/MINUTE)

Subject	Antigen	Concentration of Antigen				
		0 (Control)	1:1000	1:100	1:10	Undiluted
RM (M)	CAD-6	2450	2982	2763	5394	4924
	M	2368	1392	1704	1382	2046
	M+L	2485	2016	1963	2801	6031
	PP	2895	2973	1329	1900	2474
	CF	2189	2684	6948	6381	8540
	WC	2180	2984	2703	2623	5050
	Latex	2098	2132	2261	1983	2323
TH (M)	CAD-6	1996	2901	4414	9479	4880
	M	2174	2767	1542	1343	1433
	M+L	1914	1474	1410	3042	3928
	PP	1860	2747	1982	2843	4121
	CF	2409	1382	1042	1936	5834
MP (F)	CAD-6	2731	4699	5827	8873	10930
	M	3033	3217	3927	4768	5031
	M+L	2973	13017	16950	16465	12784
	CF	3235	10379	16463	19768	15577
	WC	1984	3204	2180	4762	5357
	Latex	1826	1902	1823	1840	1873

TABLE 1 (continued)

Subject	Antigen	Concentration of Antigen				
		0 (Control)	1:1000	1:100	1:10	Undiluted
JR (M)	CAD-6	1663	873	959	943	2039
	M	1280	1871	1273	1367	1138
	PP	1723	1913	1430	1367	4549
	CF	1333	1630	1237	3229	4533
	WC	1558	1992	1632	1670	1932
MF (F)	CAD-6	7549	23349	20385	23566	24158
	M	7476	14694	13921	10267	13973
	M+L	7880	14606	16548	15760	13444
	PP	7981	10841	12376	10437	10616
	CF	6221	16586	16180	18848	48529
	WC	7220	12901	12327	13228	15885
GS (M)	CAD-6	6310	11596	13904	21485	1153
	M	7746	10890	4747	3924	3568
	M+L	6215	14924	8442	7631	6568
	PP	7230	7389	12936	8459	6729
	CF	6919	9771	12848	14543	13891
	WC	5822	9439	9460	9810	12812
EM (M)	CAD-6	925	1681	1607	24050	1430
	M	802	890	1236	722	782
	M+L	889	880	1085	690	651
	PP	903	925	1084	930	882
	CF	923	897	928	3416	1214

TABLE 1 (continued)

Subject	Antigen	Concentration of Antigen				
		0 (Control)	1:1000	1:100	1:10	Undiluted
MG (M)	CAD-6	4582	4021	3628	8506	13406
	M	5096	3921	3627	3608	6673
	M+L	4400	3010	3552	7536	10560
	CF	4075	4470	3725	13052	15645
	WC	4172	3026	2764	6641	12521
SG (F)	CAD-6	9065	3122	3231	4298	18130
	M	8797	4435	3231	4298	10130
	M+L	6765	4155	3708	5247	17589
	PP	1723	1913	1430	2440	5275
	CF	1342	3163	2819	7901	12749
	WC	1558	1992	1632	1401	1670
	Latex	7344	1156	1036	1885	7327
LD (F)	CAD-6	6580	5253	6635	9928	15330
	M	8273	2361	2259	1877	10272
	M+L	7572	2155	2657	10300	16152
	Latex	7233	1833	929	1477	6483
AF (M)	CAD-6	6580	5253	6639	9928	15330
	M	5806	4062	4694	5144	9709
	M+L	4180	3513	4670	13376	17974
	PP	6506	6335	6892	7348	8806
	CF	4789	18200	26340	42144	39270
	WC	6876	3453	8694	10260	13960

TABLE 1 (continued)

Subject	Antigen	Concentration of Antigen				
		0 (Control)	1:1000	1:100	1:10	Undiluted
MG (M)	CAD-6	4582	4021	3628	8506	13406
	M	5096	3921	3627	3608	6673
	M+L	4400	3010	3552	7536	10560
	CF	4075	4470	3725	13052	15645
	WC	4172	3026	2764	6641	12521
SG (F)	CAD-6	9065	3122	3231	4298	18130
	M	8797	4435	3231	4298	10130
	M+L	6765	4155	3708	5247	17589
	PP	1723	1913	1430	2440	5275
	CF	1342	3163	2819	7901	12749
	WC	1558	1992	1632	1401	1670
	Latex	7344	1156	1036	1885	7327
LD (F)	CAD-6	6580	5253	6635	9928	15330
	M	8273	2361	2259	1877	10272
	M+L	7572	2155	2657	10300	16152
	Latex	7233	1833	929	1477	6483
AF (M)	CAD-6	6580	5253	6639	9928	15330
	M	5806	4062	4694	5144	9709
	M+L	4180	3513	4670	13376	17974
	PP	6506	6335	6892	7348	8806
	CF	4789	18200	26340	42144	39270
	WC	6876	3453	8694	10260	13960

TABLE 1 (continued)

Subject	Antigens	Concentration of Antigen				
		0 (Control)	1:1000	1:100	1:10	Undiluted
BR (M)	CAD-6	8359	4663	5128	6732	18482
	M	5682	3192	2455	3000	9531
	M+L	7087	3164	2737	6842	15176
	CF	6164	3755	2533	4673	13852
	WC	6078	3193	4328	6742	13372
	Latex	5980	3641	4062	3980	6018
AT (F)	CAD-6	1530	1663	1792	1926	3860
	M	1540	1656	1193	1792	1426
	PP	1723	1913	1430	1781	1430
	CF	1333	2813	3163	7901	12706
	WC	1558	1992	1632	1670	4401
JL (F)	CAD-6	1607	1093	1290	2675	3545
	PP	1309	2420	1277	549	1327
	CF	1370	2037	1547	1920	6576
	WC	1207	2430	1268	2001	3301
LG (F)	CAD-6	2029	2992	3435	4029	5688
	M	2088	2235	3889	3918	2073
	M+L	3062	2773	3484	3478	6430
	PP	2540	2140	3032	3320	4017
	CF	1199	2442	3567	4525	8753
	WC	1923	2242	3425	4620	3900
	Latex	3086	2695	2439	2242	1537

TABLE 1 (continued)

Subject	Antigen	Concentration of Antigen				
		0 (Control)	1:1000	1:100	1:10	Undiluted
FD (M)	CAD-6	1803	902	2756	6490	8162
	M	3084	2504	2698	3969	2499
	M+L	1959	1725	1527	6661	7053
	CF	2901	2134	3359	5639	7833
	WC	2094	2250	3692	4188	5658
	Latex	1902	908	1089	1256	1768
RT (M)	CAD-6	3487	4869	5532	7089	9587
	M	2730	2156	2801	8198	6290
	M+L	2760	5703	5590	6891	11613
	PP	3062	2140	3032	3230	4027
	CF	4836	7843	13100	15228	10194
	WC	3230	3016	3523	6091	6513
	Latex	3919	3342	3534	2271	2201
LL (F)	CAD-6	4682	3626	4648	10343	12174
	M	3782	2105	2972	3010	3529
	M+L	3019	3664	4648	4587	10095
	CF	5000	5526	4297	5184	11058
	WC	4005	4406	6150	10418	12065
DM (M)	CAD-6	5173	4326	3987	5148	10962
	M	5287	3623	5061	5234	8763
	M+L	5077	2684	2253	2584	4412
	CF	5679	2715	3902	3142	13075

TABLE 1 (continued)

Subject	Antigen	Concentration of Antigen				
		0 (Control)	1.1000	1:100	1:10	Undiluted
JK (M)	CAD-6	8932	6109	7554	10042	11502
	M	7124	5650	6142	6402	7034
	M+L	4917	5013	3245	4813	4269
	CF	6657	3694	4004	6956	9912
	Latex	7925	3545	7332	4776	8213

M = Male

F = Female

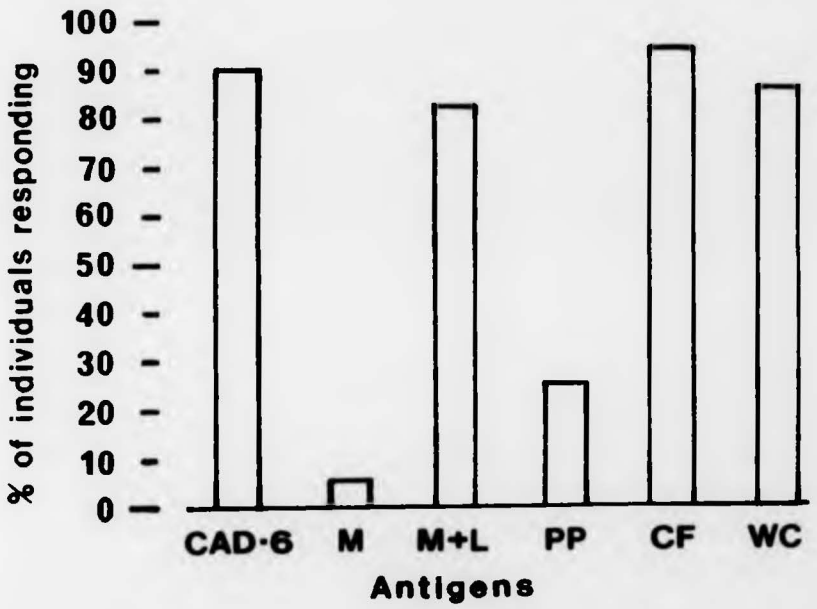


Fig. 1. % of human subjects responding to different antigens of C. albicans.

TABLE 2 NUMBER OF HUMAN SUBJECTS TESTED AND PERCENTAGE OF THOSE WHO RESPONDED TO 6 DIFFERENT ANTIGENS OF C. ALBICANS

Antigens	Tested	Responded	%
CAD-6	20	18	90.0
M	19	1	5.2
M+L	17	14	82.4
PP	12	3	25.0
CF	19	18	94.7
WC	15	13	86.6

TABLE 3 NUMBERS OF 19 SUBJECTS RESPONDING TO DIFFERENT ANTIGENS OF CANDIDA AT DIFFERENT DILUTIONS

Antigen	Final concentration (μg/ml)			
	100	10	1	0.1
SOLUBLE				
CAD-6	16	9	4	1
M	1	1	0	0
PP	3	0	0	0
CF	17	12	8	6
	Final concentration (μg/ml)			
	125	12.5	1.25	0.125
PARTICULATE				
M+L	12	5	3	2
	Concentration (cells) /ml			
	10^4	10^3	10^2	10
INTACT CELLS				
WC	13	4	0	0

TABLE 4 STIMULATION INDICES OF PERIPHERAL LYMPHOCYTES OF 20 HUMAN SUBJECTS TO SIX ANTIGENS OF CANDIDA

Subjects	CAD-6	M	M+L	PP	CF	WC
RM	2.0-2.2		2.4		2.9-3.9	2.3
TH	2.2-4.7		2.0	2.2	2.4	
MP	2.1-4.0		4.3-5.7		3.2-6.1	2.4-2.7
JR				2.6	2.4-3.4	
MF	2.7-3.2		2.0-2.1		2.6-7.8	2.2
GS	2.2-3.4		2.4		2.0-2.1	2.0
EM	2.6				3.7	
MG	2.9		2.4		3.2-3.8	3.0
SG	2.0		2.6	3.0	2.1-9.5	
LD	2.2		2.1			
AF	2.3		3.2-4.3		3.8-8.8	2.0
BR	2.2		2.1		2.2	2.2
AT	2.5				2.1-9.5	2.8
JL	2.2				4.8	2.7
LG	2.8		2.1		2.0-7.3	2.0-2.4
FD	3.6-4.5		3.4-3.6		2.7	2.0-2.7
RT	2.0-2.7	2.3-3.0	2.0-4.2		2.1-3.1	2.0
LL	2.2-2.6		3.3		2.2	2.6-3.0
DM	2.1				2.3	
JK	No stimulation					

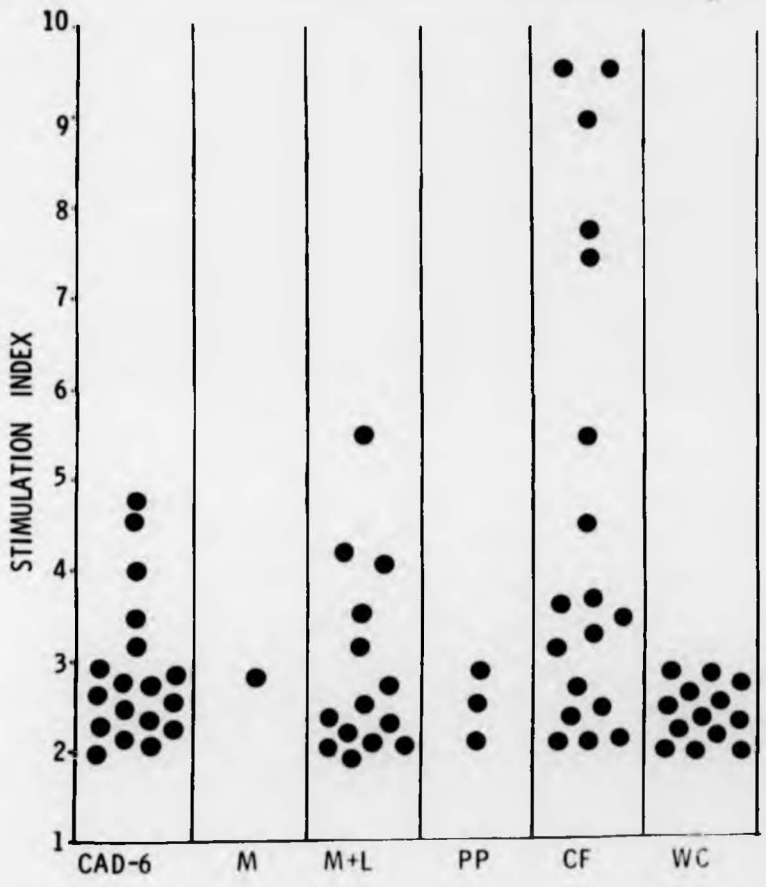


Fig. 2. Range of stimulating index of human lymphocytes tested against 6 Candida antigens.

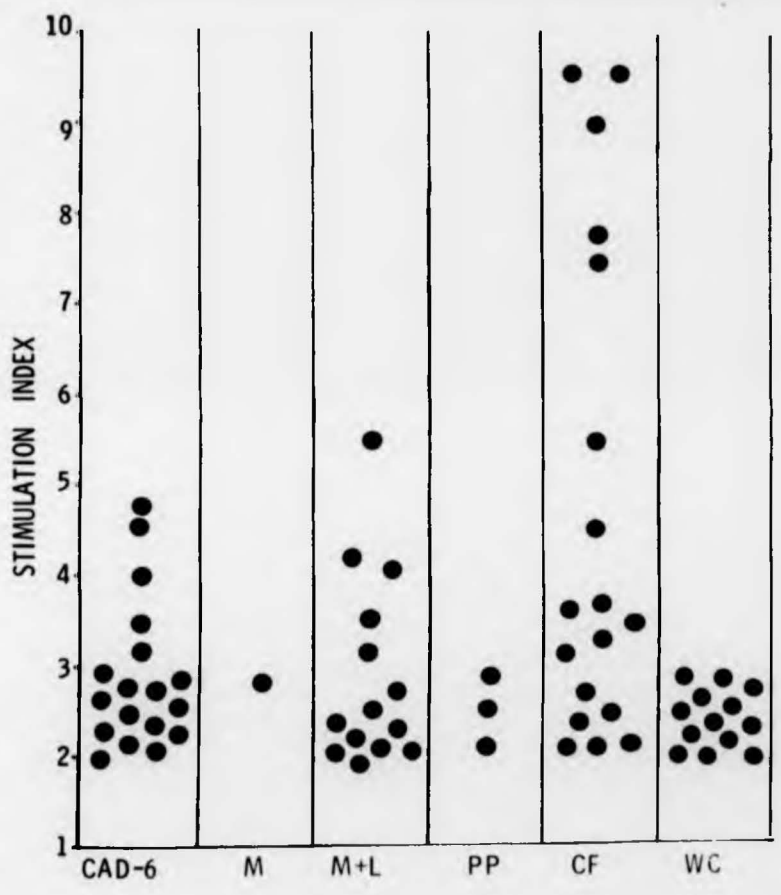


Fig. 2. Range of stimulating index of human lymphocytes tested against 6 Candida antigens.

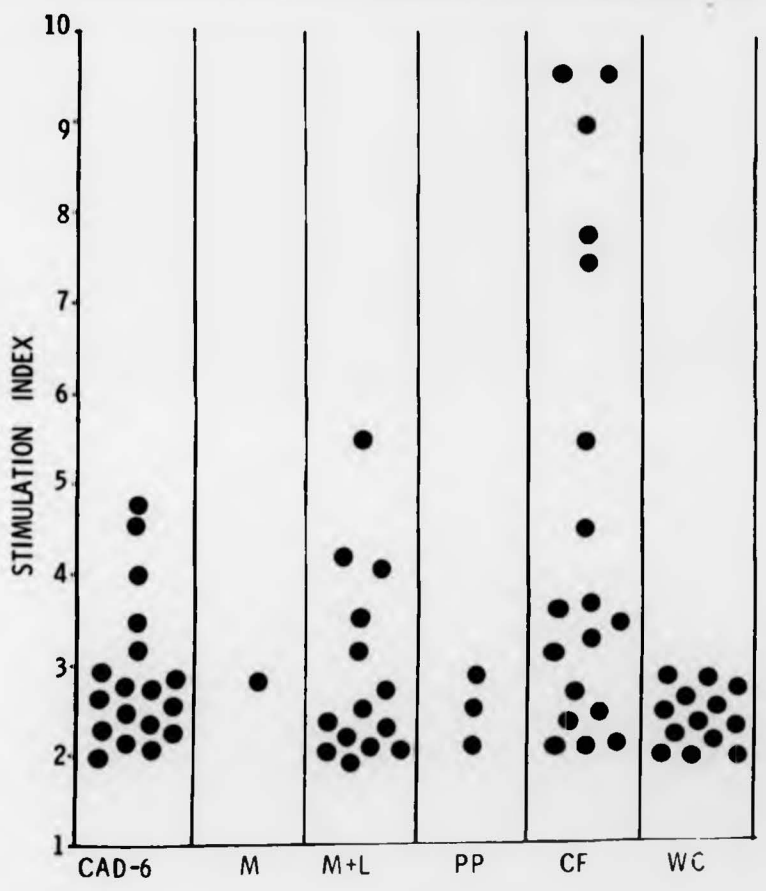


Fig. 2. Range of stimulating index of human lymphocytes tested against 6 Candida antigens.

HUMORAL RESPONSES OF NORMAL SUBJECTS TO C. ALBICANS

Determination of antibody levels for C. albicans by whole cells and particulate mannan on latex in the sera of 20 normal subjects showed that all 8 females tested had demonstrable antibody (Table 5). Titres ranged from 1:16 to 1:64. Males had lower antibody titres ranging from < 1:4 to 1:16. Seventy-five per cent had antibody for whole cell antigen and 83% for particulate mannan.

TABLE 5 DISTRIBUTION OF ANTIBODY TITRES IN 20 NORMAL HUMAN SUBJECTS TO WHOLE CELL (WC) AND MANNAN ON LATEX (M+L) ANTIGENS

Antigens	Serum dilutions						
	< 1:4	1:4	1:8	1:16	1:32	1:64	%
WC							
Females (8)				3	5		100
Males (12)	3	4	5				75
M+L							
Females (8)				3	4	1	100
Males (12)	2	3	3	4			83

ESTIMATION OF LD_{50} OF C. ALBICANS IN "TO" MICE

The mortality and survival rates of different groups of mice are recorded in Table 6. The LD_{50} of C. albicans was estimated using the method of Reed and Muench (1938). The numbers of surviving and dead animals for each concentration of inoculum are indicated in Table 7, in the second and third columns respectively. In the fourth column accumulated surviving animals were tabulated beginning with those which received the highest concentration of inoculum. In this group there were no survivors. The number of surviving animals in each of the succeeding lower concentrations of inoculum were then entered progressively towards the top of the column. Conversely the accumulative totals of dead animals were tabulated in the fifth column beginning with those which received the lowest concentration of inoculum and adding progressively the number of dead animals at each of the succeeding higher concentrations of inoculum. In the sixth column the percentage mortality is calculated as the percentage of accumulated dead mice for the particular inoculum. Thus for an inoculum concentration of 10^6 cells, an accumulation of 4 dead animals per accumulated dead plus 7 accumulated live animals gives a mortality of 4/11 or 36.4%. Likewise, the percent mortality of the inoculum contains 5×10^6 yeast cells is 8/9 or 88.8%.

The 50% proportional factor was calculated from the following ratio.

$$\frac{50\% - (\text{mortality at dilution next below})}{(\text{mortality next above}) - (\text{mortality next below})}$$

Thus: $$\frac{50 - 36.4}{88.8 - 36.4} = 0.26$$

Since dilutions were based on a logarithmic scale, the LD50 is obtained from the antilog of the difference of the log of higher dilution minus the log of lower dilution times the 50% proportional factor plus the log of the lower dilution.

$$LD_{50} = \text{antilog } ((\log 5 \times 10^6 - \log) \times 0.26 + \log 10^6)$$

$$\text{antilog } ((6.6990 - 6.0000) \times 0.26 + 60000)$$

$$\text{antilog of } 6.1817 = 1.52 \times 10^6$$

The LD_{50} was calculated to be 1.52×10^6 cells of C. albicans. The calculation was also determined graphically (Fig. 3).

Impression smears and colony counts from different organs studied revealed that kidneys were the most populated organs (Fig. 4, 5 and 6). Histological preparations revealed numerous abscesses with a cellular reaction consisting mostly of polymorphonuclear cells (Fig. 7, 8 and 9).

TABLE 6 GROUPS OF TO MICE INJECTED WITH DIFFERENT DOSES OF CANDIDA BLASTOSPORES FOR DETERMINATION OF LD_{50}

No. of mice inoculated	No. of Candida blastospores injected	No. of animals dying within 30 days	Deaths of individual mice (days)
5	5×10^4	0	-
5	10^5	0	-
5	5×10^5	0	-
10	10^6	4	8-12
5	5×10^6	4	6-12
5	10^7	5	3-11
5	5×10^7	5	3-6

TABLE 7 ESTIMATION OF LD_{50} OF C. ALBICANS STRAIN NCPF 3153 IN TO MICE

Concentration of blastospores injected	Number of mice		Accumulated		Percent Mortality
	Alive	Dead	Alive	Dead	
5×10^4	5	0	22	0	0
10^5	5	0	17	0	0
5×10^5	5	0	12	0	0
10^6	6	4	7	4	36.4
5×10^6	1	4	1	8	88.8
10^7	0	5	0	13	100
5×10^7	0	5	0	18	100

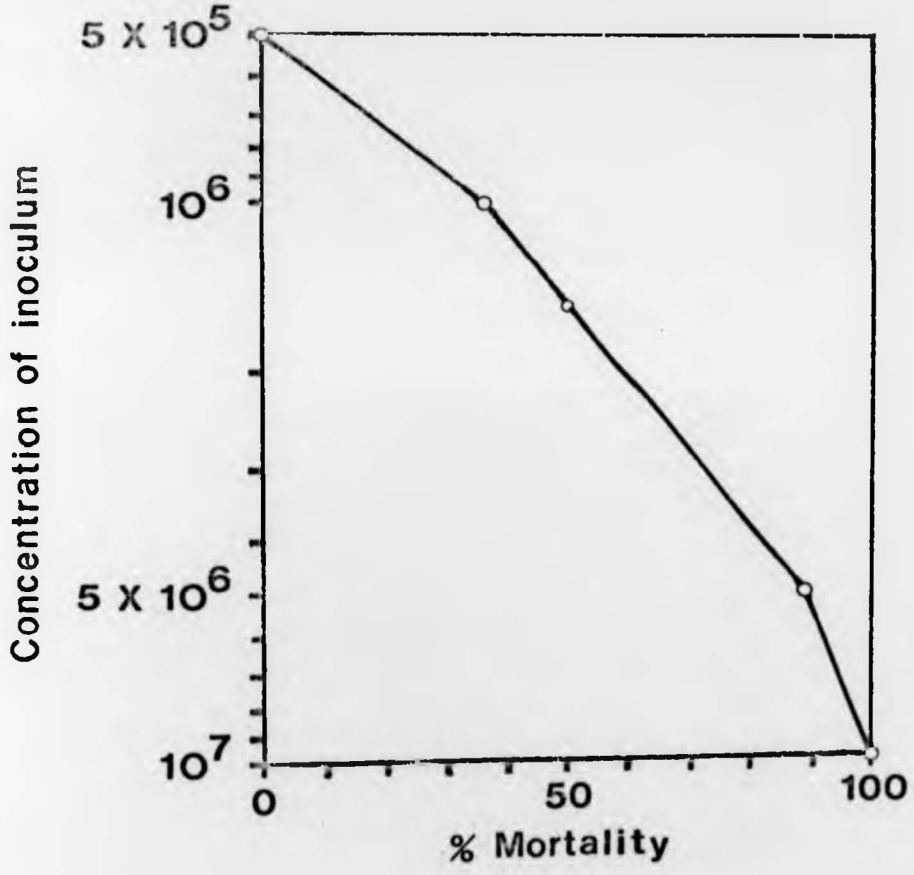


Fig. 3 Determination of LD_{50} of C. albicans in "TO" mice

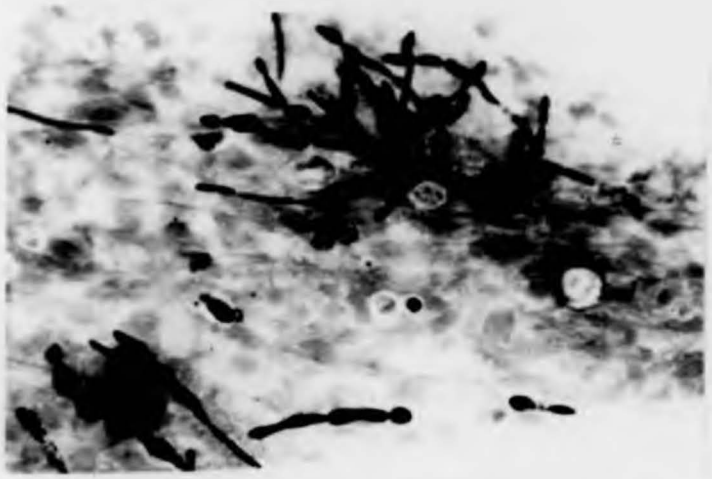


Fig. 4. Impression smear from kidney of dead mouse after challenge with 5 x 10^6 of C. albicans x 360 PAS Stain.

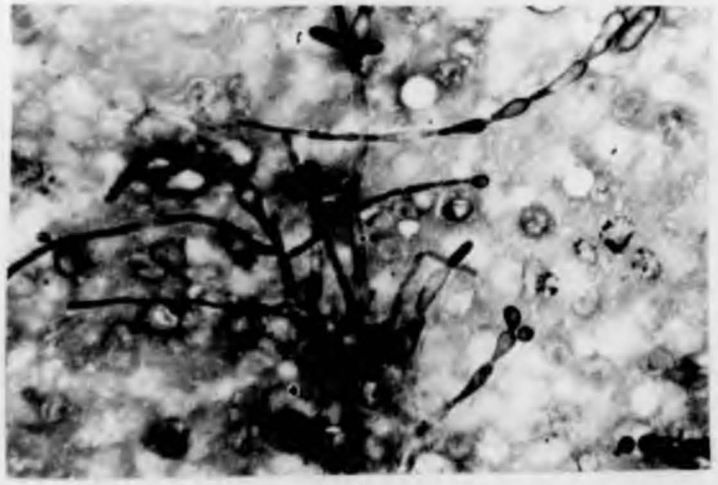


Fig. 5. Impression smear from kidney of dead mouse after challenge with 5 x 10^6 of C. albicans x 340 PAS stain.

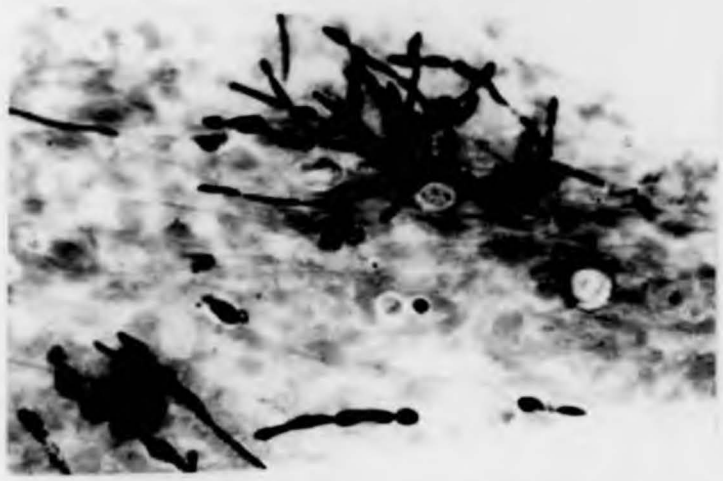


Fig. 4. Impression smear from kidney of dead mouse after challenge with 5 x 10^6 of C. albicans x 360 PAS Stain.

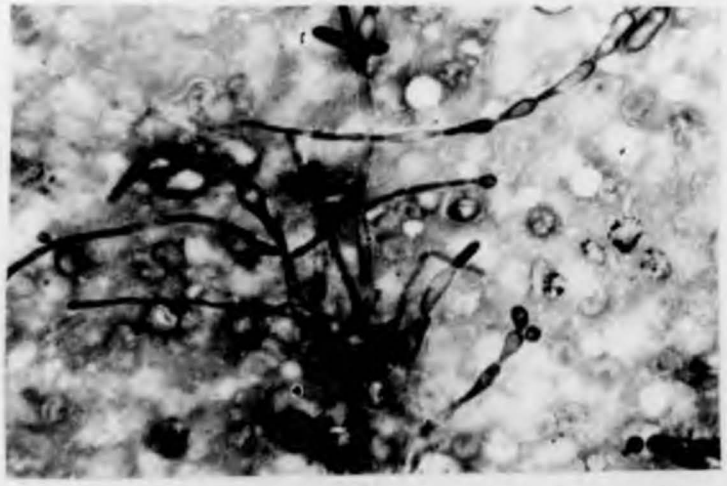


Fig. 5. Impression smear from kidney of dead mouse after challenge with 5 x 10^6 of C. albicans x 340 PAS stain.

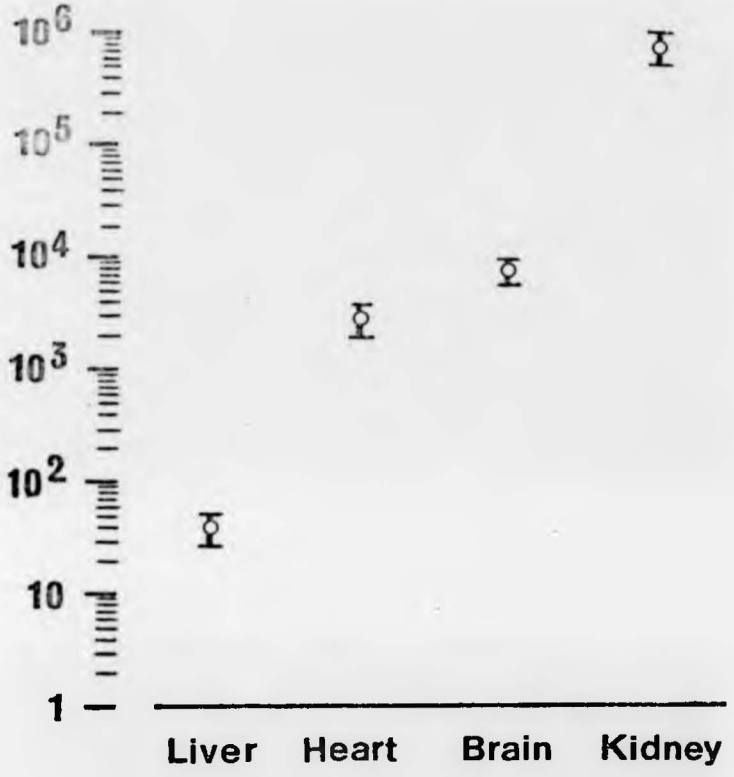


Fig. 6. Colony counts on different tissues of dead mice after challenge with 5×10^6 of C. albicans

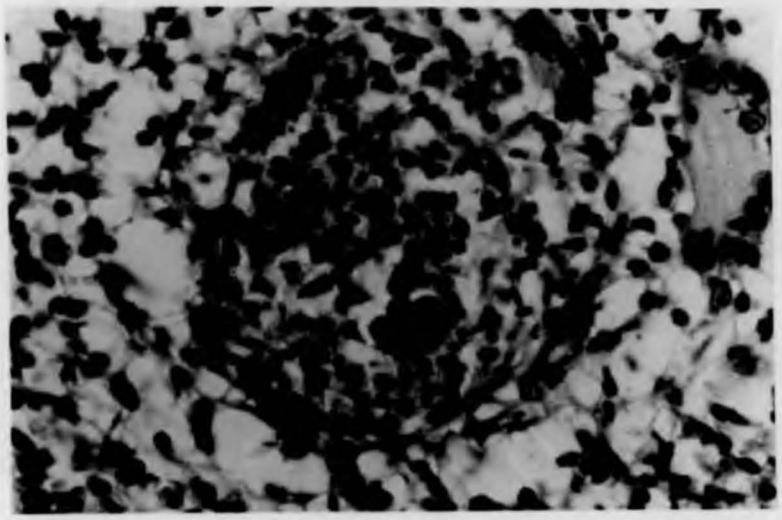


Fig. 7. Section of kidney of mouse dead after challenge with 5×10^6 of C. albicans. H and E stain. x 370.

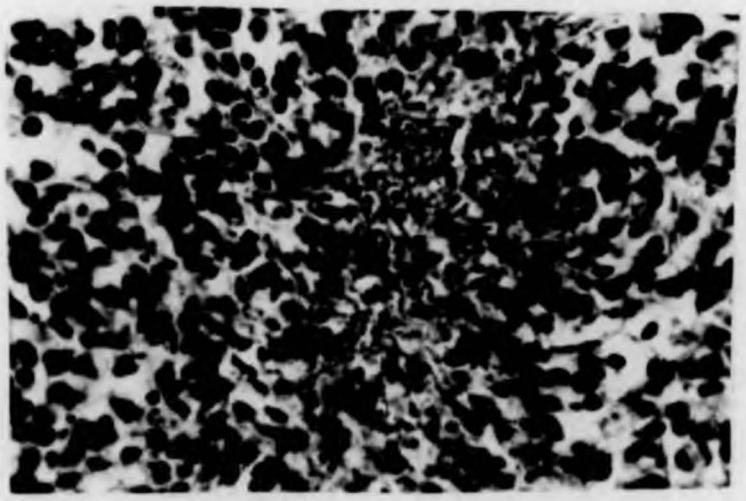


Fig. 8. Section from kidney of dead mouse after challenge with 5×10^6 of C. albicans showing an abscess. H and E stain. x 370.

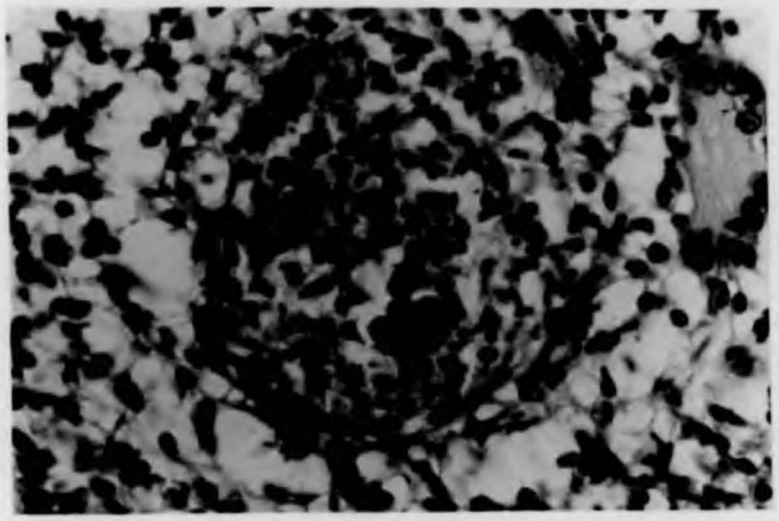


Fig. 7. Section of kidney of mouse dead after challenge with 5×10^6 of C. albicans. H and E stain. x 370.

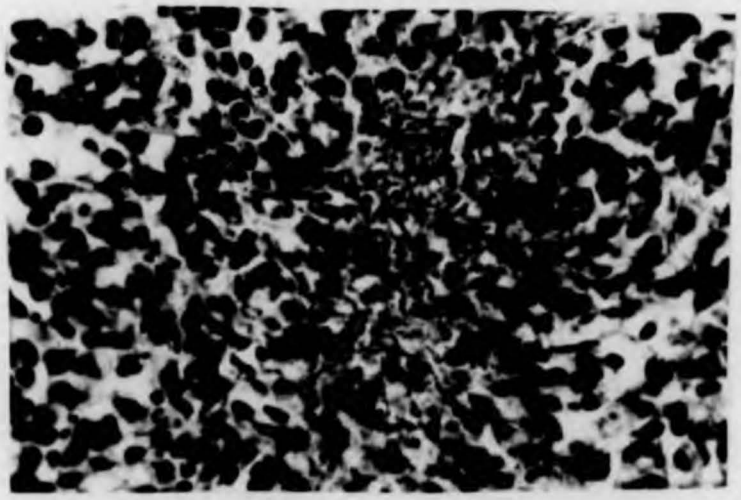


Fig. 8. Section from kidney of dead mouse after challenge with 5×10^6 of C. albicans showing an abscess. H and E stain. x 370.

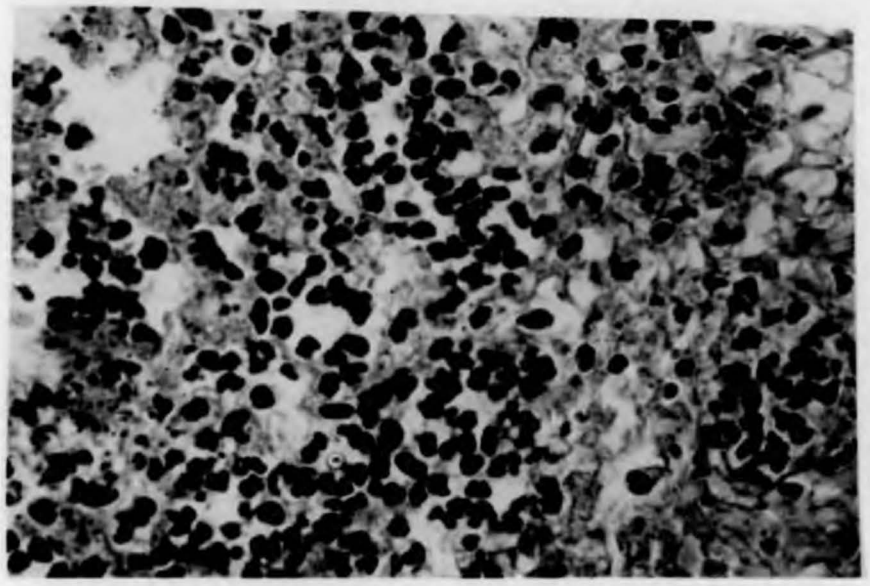


Fig. 9. Section of kidney from dead mice after challenge with 5×10^6 cells of C. albicans with cellular reaction mostly of polymorphonuclear cells. H and E stain. x 380

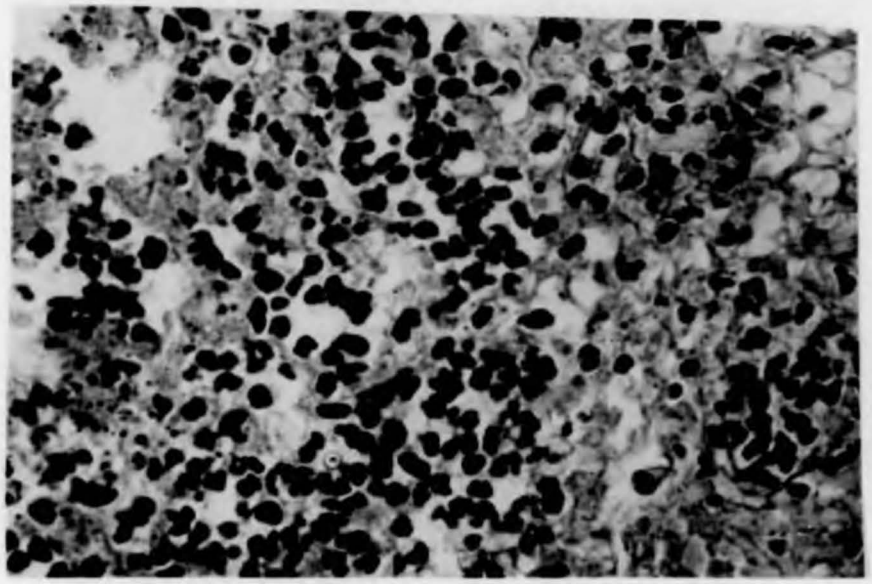


Fig. 9. Section of kidney from dead mice after challenge with 5×10^6 cells of C. albicans with cellular reaction mostly of polymorphonuclear cells. H and E stain. x 380

THIGH LESIONS IN MICE

Mice immunized with heat killed C. albicans in combination with complete Freund's adjuvant developed agglutinins as shown in Table 8. Animals inoculated intraperitoneally had higher titres than those inoculated subcutaneously.

Table 8 Agglutinating titre of pooled sera of immunized and control mice

Weeks	Control animals	Route of immunization	
		S/C	I/P
4	-	-	1:8
6	-	1:4	1:32
8	-	1:8	1:64
10	-	1:8	1:64

After the first challenge with live Candida cells both control and immunized animals developed soft localized acute inflammatory reactions which persisted for 8-10 days, before progressing to form localized palpable abscesses. These resolved spontaneously after 4 weeks. Smears from a lesion on the tenth day after infection showed cellular reactions consisting mostly of polymorphonuclear cells with few lymphocytes (Fig. 10). Histological sections showed Candida in both yeast and mycelial phase infiltrated throughout muscle fibres (Fig. 11 and 12).

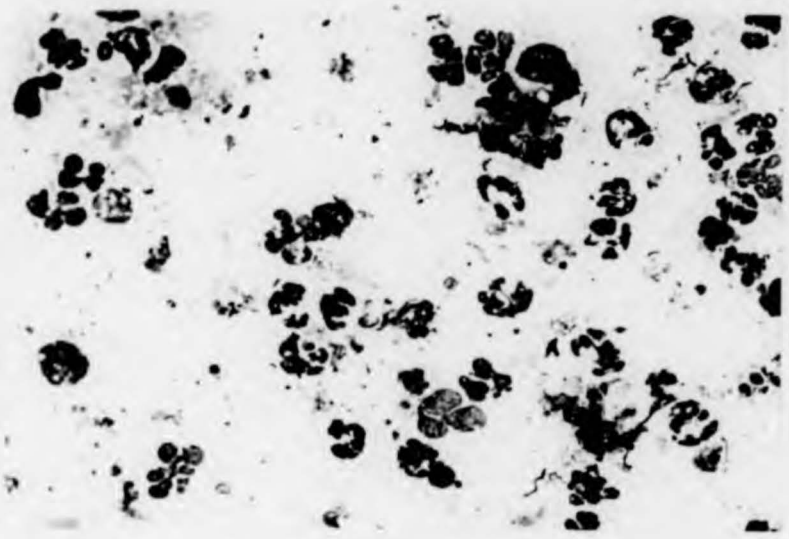


Fig. 10. Smear from thigh lesion 10 days after first challenge with C. albicans with cellular reaction mostly of PMN. Giemsa stain. x 425.

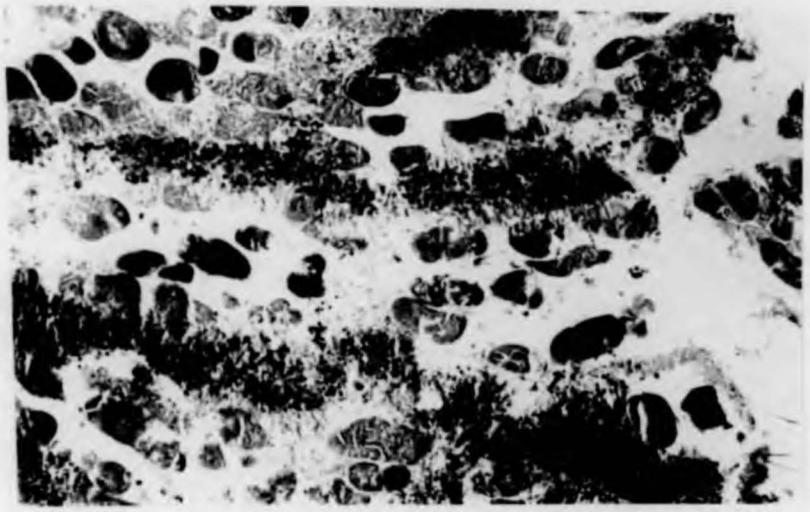


Fig. 11. Thigh lesion in mouse with C. albicans. PAS stain. x 105.

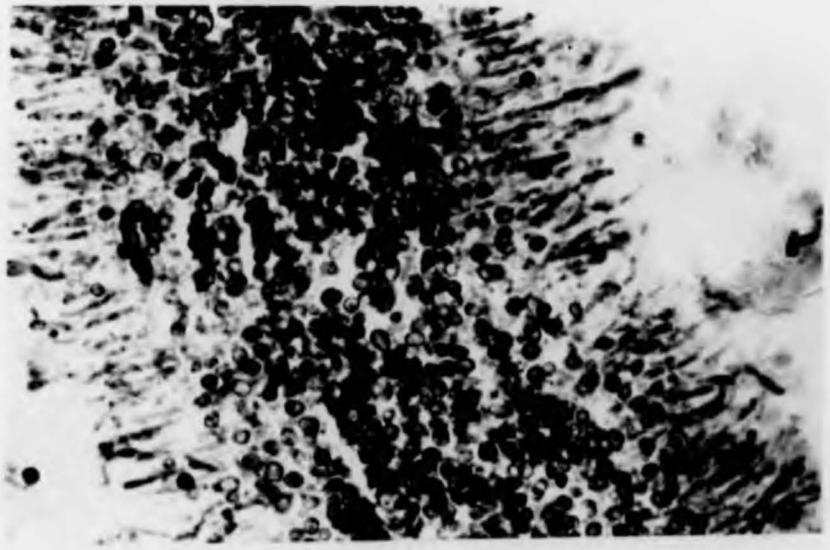


Fig. 12. Thigh lesion in mouse with C. albicans. PAS stain. x 360.

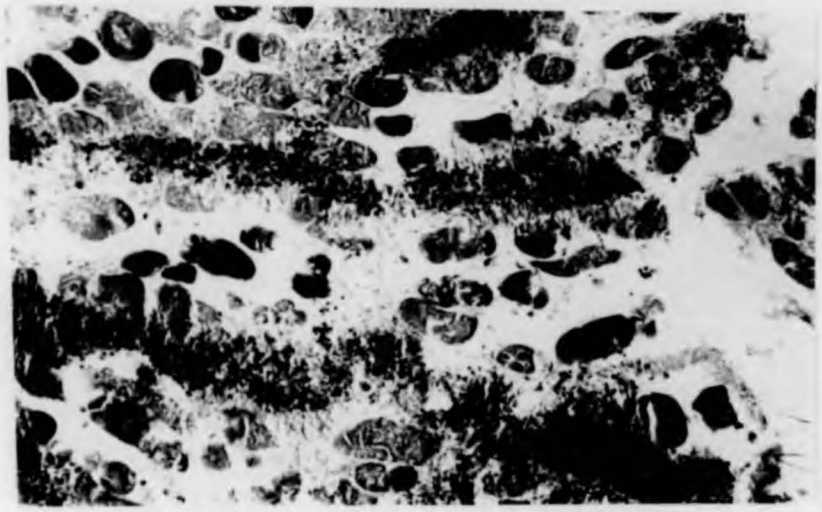


Fig. 11. Thigh lesion in mouse with C. albicans. PAS stain. x 105.

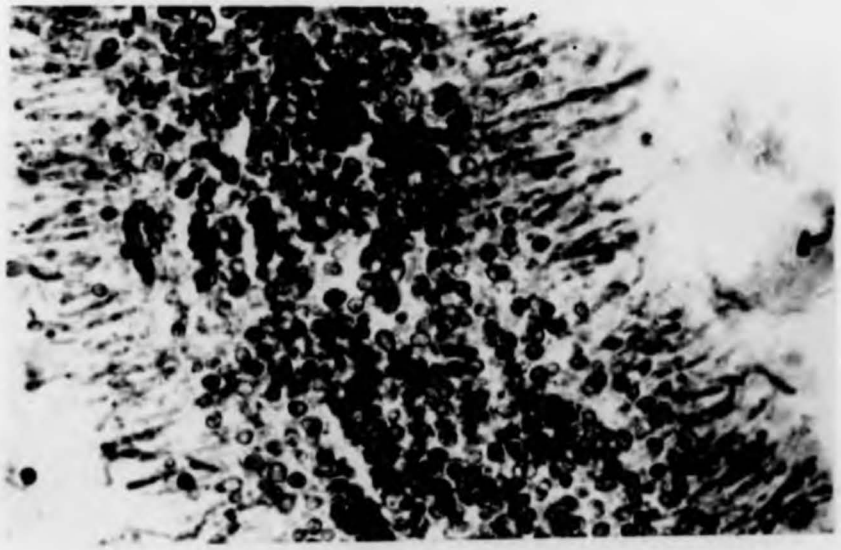


Fig. 12. Thigh lesion in mouse with C. albicans. PAS stain. x 360.

Spleens of test animals were enlarged as compared to controls (Fig. 13). Weights of the spleen from animals immunized by the i/p route were consistently heavier than s/c immunized mice. Both groups had spleens which were appreciably heavier than those from control animals (Table 9).

Table 9. Spleen weights (gm) of individual immunized and control mice at the time of killing

Weeks	Control	Immunized	
		s/c	i/p
12	0.2	0.35	0.5
12	0.2	0.42	0.8
16	0.2	0.45	0.9
20	0.25	0.48	1.0
20	0.25	0.5	1.0
Average	0.22	0.44	0.84

Tissue sections of spleen showed hyperplasia of both red and white pulp with predominance of large mononuclear cells in the test animals (Fig. 14). The second thigh challenge one month later resulted in an infection very similar to the first lesion except that the inflammatory response of test animals subsided 2-3 days earlier than the controls. No difference in cell response was

noticed in smears from the lesions. Polymorphonuclear cells were the predominant cells (Fig. 15).

Following the third challenge, a month after the second, sequestration of the abscess in the test animal was appreciably accelerated. By the fifth day the abscess had become walled off while in the control it was still active and spreading. Candida appeared mostly in the yeast phase in the test animal while in the control animals it was mostly in the mycelial phase (Fig. 16, 17, 18, 19 and 20).

Fig. 13. Spleens of immunized and then challenged mice with C. albicans x 2
Left (i/p) Middle (s/c) Right (control)

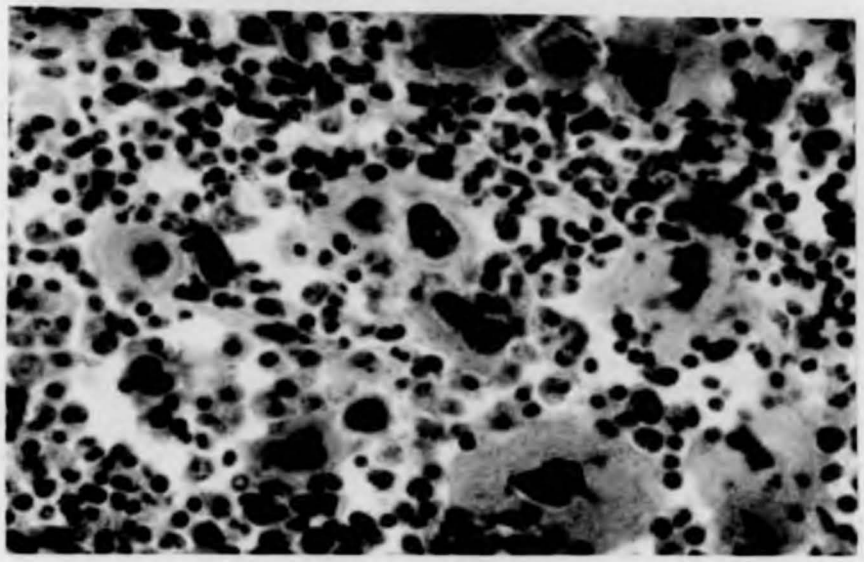


Fig. 14. Section of spleen in i/p immunized mice showing predominance of mononuclear cells H and E stain. x 410

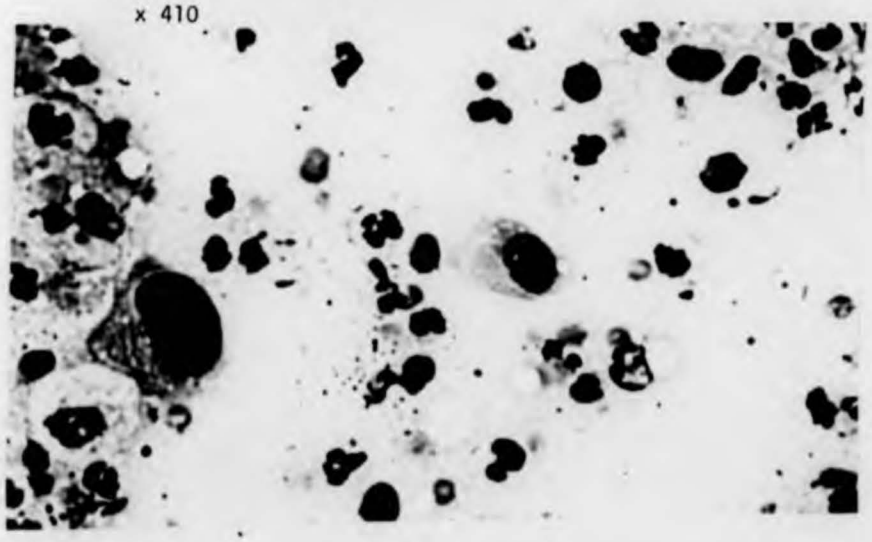


Fig. 15. Smear from lesion 10 days after the second challenge. Giemsa stain. x 450

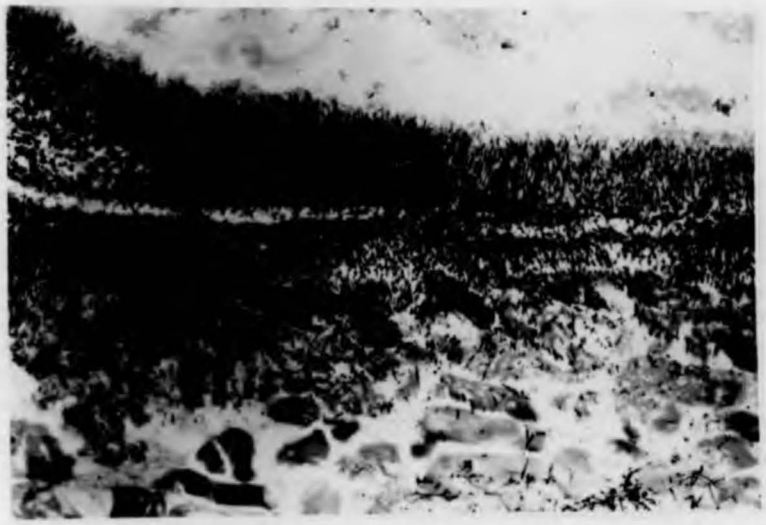


Fig. 16. Section of thigh lesion in control mouse 5 days after infection with C. albicans. PAS stain. x95.

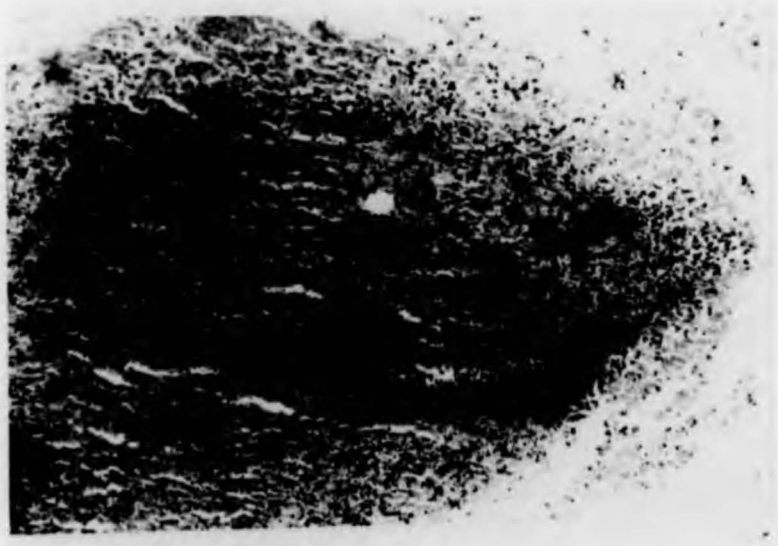


Fig. 17. Section of thigh lesion in immunized mouse 5 days after infection with C. albicans. PAS stain. x92.

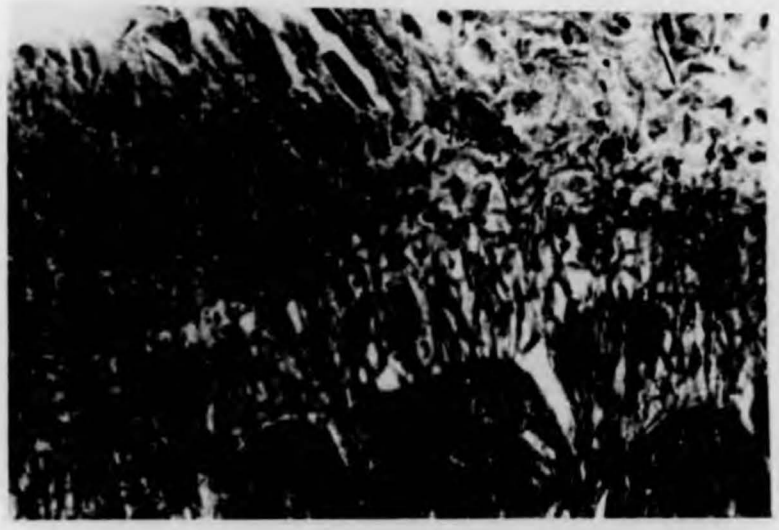


Fig. 18. Section of thigh lesion in control mouse 5 days after infection with C. albicans mostly in mycelial phase. PAS stain. x380.

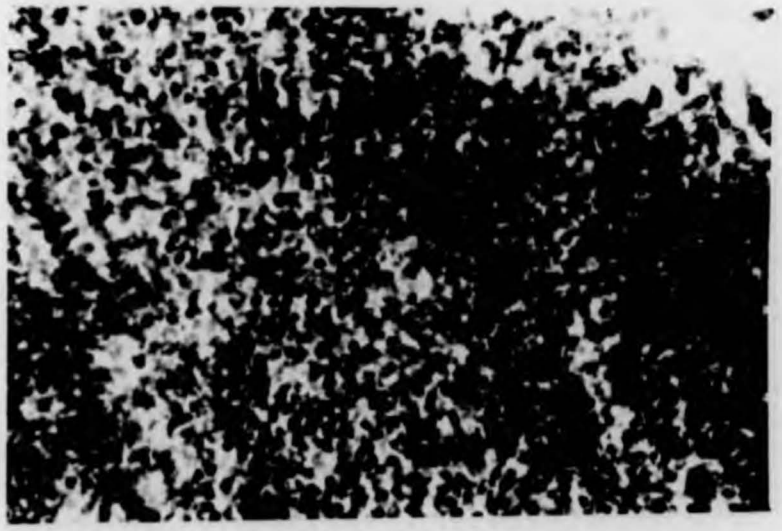


Fig. 19. Section of thigh lesion in immunized mouse 5 days after infection with C. albicans mostly in yeast phase. PAS stain. x370.

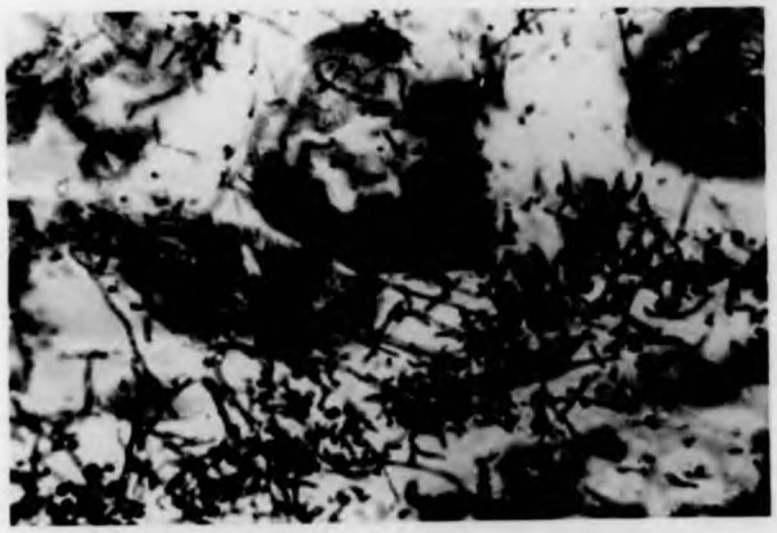


Fig. 20. Section of thigh lesion in control mouse 5 days after infection with C. albicans showing the dominance of mycelial phase. PAS stain. x380.

ACQUIRED RESISTANCE OF MICE TO C. ALBICANS

Mice immunized with different antigen preparations of C. albicans showed different degrees of resistance both in survival time and survival rate when challenged with lethal doses of the live organism (Table 10). The effectiveness of the antigens in protecting immunized animals were in the following order:

CAD-6 > M+L > CF > PP > WC(A) = M > WC(K)

Heat killed vaccine (WC(K)) did not confer any measurable protection to the immunized mice and their mortality rate differed in no way from that of the controls.

Serological studies of the animals just before challenge with live organisms showed that their agglutinin titres ranged from 1:4 to 1:64. The group immunized with heat killed Candida had the highest agglutinin titre. The precipitin reactions as determined by CIE were directed principally towards the CAD-6 and purified protein antigens. There was no detectable reaction to mannan whether or not it was adsorbed onto latex (Table 11).

Surviving animals showed agglutinin titres which ranged from 1:4 to 1:16, and precipitins directed primarily against CAD-6 and PP antigens. None of the groups showed any anti-mannan precipitins which could be detected by CIE or DD methods (Table 12).

Further tests involving treatment of mannan with pronase to destroy traces of protein and treatment of purified protein to oxidize traces of mannan did not influence the results and confirmed that this strain of mice did not produce precipitins against mannan.

The significance of prolonged survival time in mice immunized with various antigens after challenge with a lethal dose of live C. albicans was determined. By application of the Mann Whitney U test it was shown that P values for CAD-6, M+L, PP and CF were < 0.05 while M, WC(K) and WC(A) had a P value > 0.05 (Table 13).

With regard to the number of surviving animals immunized with various antigens after challenge with a lethal dose of live C. albicans by applying the Fisher's exact probability test it was shown that CAD-6, M+L, CF had P values < 0.05 in contrast to M, PP, WC(K) and WC(A) antigens whose P values were > 0.05 (Table 14).

TABLE 10 MORTALITY OF IMMUNIZED AND CONTROL MICE CHALLENGED WITH 4×10^6 CELLS OF C. ALBICANS

Duration of infection	Control Animals	Antigens						
		CAD-6	M	M+L	PP	CF	WC(K)	WC(A)
Day 0								
1								
2								
3								
4								
5								
6	1		1				2	1
7	1		1				1	1
8	1		1				1	1
9	2		1			1	1	2
10	2					1	2	1
11			1	1	1	1	1	1
12	1		1	1		1	1	
13		1		1	1	1	1	
14	1	1						
15								
16	1							
17					1			
18								
19		1						
20					1			
21			1		1			
22					1			
23								
24								
25								
26								
27								
28				1				
29								
30								
31								
Total Deaths	10	3	7	4	6	7	10	7

TABLE 10 MORTALITY OF IMMUNIZED AND CONTROL MICE CHALLENGED WITH 4×10^6 CELLS OF C. ALBICANS

Duration of infection	Control Animals	Antigens						
		CAD-6	M	M+L	PP	CF	WC(K)	WC(A)
Day 0								
1								
2								
3								
4								
5								
6	1		1				2	1
7	1		1				1	1
8	1		1				1	1
9	2		1			1	1	2
10	2					1	2	1
11			1	1	1	1	1	1
12	1		1	1		1	1	
13		1		1	1	1	1	
14	1	1						
15								
16	1							
17								
18					1			
19		1						
20								
21					1			
22			1		1			
23					1			
24								
25								
26								
27								
28				1				
29								
30								
31								
Total Deaths	10	3	7	4	6	7	10	7

TABLE 11 ANTIBODY RESPONSES IN POOLED SERA FROM MICE VACCINATED WITH DIFFERENT ANTIGEN PREPARATIONS OF C. ALBICANS

Antigens	Precipitins*			Agglutinins
	CAD-6	M	PP	
CAD-6	2 lines	-	2 lines	1:4
M	-	-	-	< 1:4
M+L	-	-	-	< 1:4
PP	2 lines	-	3 lines	1:4
CF	-	-	-	< 1:4
WC(K)	-	-	-	1:64
WC(A)	-	-	-	1:8

* detected by CIE

TABLE 11 ANTIBODY RESPONSES IN POOLED SERA FROM MICE VACCINATED WITH DIFFERENT ANTIGEN PREPARATIONS OF C. ALBICANS

Antigens	Precipitins*			Agglutinins
	CAD-6	M	PP	
CAD-6	2 lines	-	2 lines	1:4
M	-	-	-	< 1:4
M+L	-	-	-	< 1:4
PP	2 lines	-	3 lines	1:4
CF	-	-	-	< 1:4
WC(K)	-	-	-	1:64
WC(A)	-	-	-	1:8

* detected by CIE

TABLE 12 SEROLOGICAL STATUS OF SURVIVING MICE IN EACH GROUP 31 DAYS AFTER CHALLENGE WITH 4 x 10^6 LIVE CELLS OF C. ALBICANS

Immunizing antigen	Survival rate %	Precipitins*			Agglutinins	Spleen weight Gm
		CAD-6	M	PP		
CAD-6	70	2 lines	-	3 lines	1:8	0.38
M	30	-	-	-	1:4	0.30
M+L	60	1 line	-	1 line	1:4	0.38
PP	40	1 line	-	3 lines	1:16	0.28
CF	50	1 line	-	1 line	1:4	0.42
WC(A)	30	1 line	-	1 line	1:16	0.37
Control	0	-	-	-	-	0.15

* detected by CIE

The Mann-Whitney U ranking test was used to compare the individual survival times of the mice in the experimental groups with those in the control group (Von Fraunhofer and Murray, 1976). The survival times of the control mice (N_1) were placed in the first line beginning with the shortest and proceeding in ascending order and those of the experimental mice (N_2) in the second line. On the third line all the survival times were combined and on the fourth line a ranking order was made beginning with 1 for the smallest survival time, 2 for the next and so on. When two survival times were tied the average rank was given to both. On the fifth and sixth lines the ranks were identified as belonging to group N_1 or N_2 and their sums were designated as R_1 or R_2 (see example below).

Control	(N_1)	6	7	8	9	9	10	10	12	14	16
CAD-6	(N_2)	13	14	19	31	31	31	31	31	31	31

Combined	6	7	8	9	9	10	10	12	13	14	14	16	31 x 7
Ranking	1	2	3	4.5	4.5	6.5	6.5	8	9	10.5	10.5	12	13	17 x 7

N_1 1 + 2 + 3 + 4.5 + 4.5 + 6.5 + 6.5 + 8 + 10.5 + 12 = 58.5 (R_1)

N_2 9 + 10.5 + 13 + 17 + 17 + 17 + 17 + 17 + 17 + 17 = 151.5 (R_2)

The following formulas were then calculated:

$$U_1 = n_1 n_2 + \frac{n_1(n_2+1)}{2} - R_1$$

$$U_2 = n_1 n_2 + \frac{n_2(n_2+1)}{2} - R_2$$

Where n_1 is the number of mice in group N_1 = 10

n_2 is the number of mice in group N_2 = 10

$$U_1 = 10 \times 10 + \frac{10(10+1)}{2} - 58.5 = 96.5$$

$$U_2 = 10 \times 10 + \frac{10(10+1)}{2} - 151.5 = 3.5$$

The smaller value, U_2 (3.5), was then converted to probability by the conversion table given in the text. 3.5 = 0.02.

The Mann-Whitney U ranking test was used to compare the individual survival times of the mice in the experimental groups with those in the control group (Von Fraunhofer and Murray, 1976). The survival times of the control mice (N_1) were placed in the first line beginning with the shortest and proceeding in ascending order and those of the experimental mice (N_2) in the second line. On the third line all the survival times were combined and on the fourth line a ranking order was made beginning with 1 for the smallest survival time, 2 for the next and so on. When two survival times were tied the average rank was given to both. On the fifth and sixth lines the ranks were identified as belonging to group N_1 or N_2 and their sums were designated as R_1 or R_2 (see example below).

Control (N_1)	6	7	8	9	9	10	10	12	14	16	
CAD-6 (N_2)	13	14	19	31	31	31	31	31	31	31	
Combined	6	7	8	9	9	10	10	12	13	14	14	16	31 x 7
Ranking	1	2	3	4.5	4.5	6.5	6.5	8	9	10.5	10.5	12	13	17 x 7

N_1 1 + 2 + 3 + 4.5 + 4.5 + 6.5 + 6.5 + 8 + 10.5 + 12 = 58.5 (R_1)

N_2 9 + 10.5 + 13 + 17 + 17 + 17 + 17 + 17 + 17 + 17 = 151.5 (R_2)

The following formulas were then calculated:

$$U_1 = n_1 n_2 + \frac{n_1(n_2+1)}{2} - R_1$$

$$U_2 = n_1 n_2 + \frac{n_2(n_2+1)}{2} - R_2$$

Where n_1 is the number of mice in group N_1 = 10

n_2 is the number of mice in group N_2 = 10

$$U_1 = 10 \times 10 + \frac{10(10+1)}{2} - 58.5 = 96.5$$

$$U_2 = 10 \times 10 + \frac{10(10+1)}{2} - 151.5 = 3.5$$

The smaller value, U_2 (3.5), was then converted to probability by the conversion table given in the text. 3.5 = 0.02.

TABLE 13 SUMMARY OF THE MANN-WHITNEY U TEST APPLIED TO THE SURVIVAL TIME OF MICE IMMUNIZED WITH VARIOUS ANTIGENS, AFTER CHALLENGE WITH A LETHAL DOSE OF LIVE C. ALBICANS

Antigen	U_1	U_2	Probability
CAD-6	96.5	3.5	$P < 0.02$
M	63	37	$P > 0.05$
M+L	92.5	7.5	$P < 0.02$
PP	95	5	$P < 0.02$
CF	82.5	17.5	$P < 0.02$
WCK	43.5	56.5	$P > 0.05$
WCA	55.5	44.5	$P > 0.05$

A comparison of the numbers of surviving and dead animals of each experimental groups vs. the control group was made using Fisher's exact probability test (Swinscow, 1976).

The number of living and dead animals in the control group and the experimental group were arranged in a box as follows (using CAD-6 antigen as an example).

a	b	r_1
c	d	r_2
s_1	s_2	N

Where a = the number of surviving control mice (0)

b = the number of dead control mice (10)

c = the number of surviving experimental mice (7)

d = the number of dead experimental mice (3)

r_1 = total number of control mice (10)

r_2 = total number of experimental mice (10)

s_1 = total number of surviving mice (7)

s_2 = total number of dead mice (13)

N = total number of all mice in test (20)

These numbers were put into the following formula:

$$P_o = \frac{r_1!\ r_2!\ s_1!\ s_2!}{N!\ a!\ b!\ c!\ d!}$$

$$P_o = \frac{10!\ 10!\ 7!\ 13!}{20!\ 0!\ 10!\ 7!\ 3!}$$

$$= \frac{(3.6288 \times 10^6)\ (6.2270 \times 10^9)}{(2.4329 \times 10^{18}) \times 6}$$

$$= 1.5479 \times 10^{-3} = 0.0015479$$

$$P = 2P_o = 2 \times 0.0015479$$

$$P = 0.031$$

TABLE 14 SUMMARY OF THE EXACT PROBABILITY TEST OF FISHER ET AL. (SWINSCOW 1976) APPLIED TO THE NUMBERS OF SURVIVING AND DEAD MICE IMMUNIZED WITH VARIOUS ANTIGENS AFTER CHALLENGE WITH A LETHAL DOSE OF LIVE C. ALBICANS

Antigens	Number dead	Number alive	Probability
Control	10	0	-
CAD-6	3	7	0.0031
M	7	3	0.21
M+L	4	6	0.011
PP	6	4	0.087
CF	5	5	0.033
WCK	10	0	1.00
WCA	7	3	0.21

DISCUSSION

Substances which stimulate human peripheral lymphocytes are divided into two groups. One consists of non-specific mitogens such as phytohaemagglutinin (PHA) and concanavalin A (Con A). These induce measurable stimulation of the lymphocytes from most adult subjects as well as those from the newborn, and are entirely independent of immunization. The other group of mitogens consists of antigens such as purified protein derivative (PPD) or extracts of Candida. These are specific, acting only on the lymphocytes of sensitized individuals. Antigen-induced lymphocyte transformation represents a secondary response in vitro: it is not noted in cells from the newborn.

Lymphocytes responding to such stimuli possess appropriate receptors on their surfaces specific for the stimulating antigen. The first step in lymphocyte transformation is recognition of the stimulating antigen and its active association with these receptors. The next step is activation which is triggered by the recognition phase. Changes are brought about in biosynthesis energy metabolism and cellular morphology of the activated lymphocytes. Following activation, the sensitized lymphocytes release into the culture fluid substances known as lymphokines causing the non-sensitized lymphocytes to undergo blast formation. Lymphocyte transformation is measured in vitro by culturing the cells in the presence of a labelled precursor of nucleic acid e.g. tritiated thymidine for DNA. Transformation is revealed and can be quantitatively determined by measuring the increased amounts of incorporation of tritiated thymidine into cellular DNA.

A stimulating antigen must be specific, it must contain appropriate antigenic determinants and it must be presented in such a form to be recognized by the receptors of the sensitized lymphocytes.

In this study the lymphocytes of most human subjects were found to respond to the crude, particulate or culture filtrate antigens of Candida albicans. Other investigators have already shown that lymphocytes from a high percentage of the normal population are stimulated by Candida antigen (Alford, 1973; Foroozanfar et al., 1974 and Frisk et al., 1974).

In comparing the stimulatory effect of the six different Candida antigens used in this study it was found that culture filtrate, crude antigen and particulate mannan were superior both in potency and magnitude of response, in stimulating lymphocytes from healthy subjects. Intact, heat killed cells of C. albicans also caused some stimulation but to a lesser degree. The least potent antigens in the system studied were cytoplasmic proteins and soluble mannan. They did stimulate lymphocytes to a small degree but the stimulation index never exceeded the arbitrary level of significance, viz twice the control level. The fact that mannan rendered particulate by adsorption onto latex was stimulatory, suggests that the physical configuration of a stimulating antigen is of importance in inducing lymphocyte transformation in sensitized cells. A provisional conclusion from this work is that structural changes of an antigen or its mode of presentation to lymphocytes influence its stimulatory effect.

Mannan, the predominant cell wall antigen of C. albicans is a highly branched polymer of mannose consisting of an α-1-6 linked backbone with many side chains consisting of α-1-2 and α-1-3 linked oligosaccharides (Yu et al, 1967; Gorin and Spencer, 1968). Soluble mannan by itself may not be processed by macrophages and this may explain the failure of lymphocytes to transform. However mannan adsorbed onto latex particles becomes functionally particulate and its antigenic determinants become more readily available and recognized. A particulate antigen is likely to be more effective in establishing multiple bonding between antigenic determinants and lymphocyte receptors. In addition particulate bound antigens may not dissociate as rapidly as soluble antigen.

Soluble antigens are usually poor evokers of T cell responses, and according to Sultzer and Nilsson (1972) and Cohen et al. (1975) tend to induce mitogenesis of B cells. The fact that aggregated materials are better recognized by T cells was demonstrated by Moller (1969). He found that soluble antigen such as human serum albumin and mouse gamma globulin when aggregated with their corresponding antibody stimulated DNA synthesis in normal human lymphocytes cultivated in vitro, whereas antibody alone or antigen alone had no effect, or a negligible one. Zabriskie and Falk (1970) exposed tuberculin-sensitized human and rat lymphocytes to various products of the tubercle bacilli and observed a significant response of sensitized lymphocytes to intact tubercle bacilli and negligible inhibition of migration when purified protein derivative (PPD) was used. Nilsson and Moller (1972) showed that PPD-tuberculin adsorbed to bentonite always stimulated stronger lymphocyte responses than soluble PPD. The same situation could also be true for the purified

proteins of C. albicans: if they could be linked onto latex or other inert particles they might become better stimulants for sensitized lymphocytes. One problem which may be troublesome in practice is the polyvalent nature of the cytoplasmic protein of C. albicans. With so many different proteins present in such extracts there could be differences in their degree of attachment to the inert carrier and their retention of mitogenic reactivity when adsorbed onto latex particles. Since it is still not known which (if any) of the antigens detectable in cytoplasmic extract by orthodox serological tests are of diagnostic significance, it would be a major undertaking and perhaps an impracticable one to determine the adsorption and stimulatory qualities of each of the many protein or glycoprotein antigens present in a cytoplasmic extract.

The superiority of a crude and complex antigen over a pure and simple one was shown in this study. The observation that the crude antigen CAD-6 proved a good stimulant of lymphocytes was probably due to the physical as well as to the chemical complexity of its antigenic components. It can be assumed that it contains numerous antigenic determinants which are easily recognized by lymphocytes and capable of inducing multiple bonding with cell receptors. It is also likely that crude antigen contains lipids which may act as an adjuvant in the acquisition of stimulation.

Frisk et al. (1974) using different antigenic preparations of C. albicans found that glucomannan protein (GMP) of the cell wall was more potent in stimulating DNA synthesis of sensitized lymphocytes than the cytoplasmic antigen. Zawisza-Zenkteler and

Zamiechowska-Miazga (1974) studied the effects of three different antigens of C. albicans on blast formation of rabbit lymphocytes and found a relationship between the chemical composition of different antigenic fractions and their stimulatory activity. Thus the glycoprotein fraction induced transformation similar to that associated with whole cells of C. albicans while glucomannan was inactive.

The stimulatory effect of culture filtrate is considered to be due to the presence of one or more antigens which are potent elicitors of lymphocyte stimulation. Although particles such as ribosomes and membrane fragments may be present in the culture filtrate, it is nevertheless likely that the critical quality is chemical rather than physical. The fact that culture filtrate is a product of secretion and excretion of living organisms makes it a more suitable lymphocyte stimulant. This metabolic product may be similar to the one produced in vivo as the growing organism encounters the host tissue.

The comparative inferiority of the non-viable heat killed organism to stimulate in high dilution is presumed to be due to the large size of the particle which would make its processing by the macrophages difficult. However it is possible that intact whole cell lacks molecular complexity and does not have enough antigenic determinants exposed on the cell surface or perhaps the stimulating antigen is modified by heat.

Deresinski et al. (1977) studying the lymphocyte transformation of different antigenic preparations of Coccidioides immitis

found that endospore to be superior to spherules and suggested that this superiority may be due to (a) endospore possessing a different and more potent antigen, (b) having more antigen per unit area or (c) having antigen which is more readily processed or otherwise available for contact with the appropriate lymphocyte receptors. They stated that the spherules were too large to be phagocytized while the endospores were readily ingested by monocytes in culture.

Mechanisms of immunity to candidiasis in experimental animals are still the subject of investigation. The role of cell mediated immunity in resistance to infection is a matter of dispute and contradictory views have been expressed in the literature. Hurd and Drake (1953) and Winner (1956) reported failure of immunization to protect rabbits against a subsequent challenge dose. in contrast Hasenclever (1963), Dobias (1964), Mourad and Friedman (1961, 1968) reported some protection in mice as a result of active immunization. Al-Doory (1970) found an immune factor in the serum of moribund baboons infected with C. albicans but this was not found in the serum of infected rabbits. Salvin et al. (1965) considered the thymus to be an important mediator of resistance to experimental candidiasis in mice. Cutler (1976) and Rogers et al. (1976) however reported that nude (thymic-deficient) mice were more resistant to experimental infections, a phenomenon they ascribed to better, innate, non-specific defence mechanisms. Miyake et al. (1977) concluded that protection against candidiasis depends on a condition of non-immune phagocytosis and T cell-mediated immunity, and that antibody was not the principal effector mechanism in eliminating C. albicans. This contrasts with the opinion expressed by Pearsall

et al. (1978) that cell mediated immunity is not of primary importance in murine candidiasis and that humoral immunity contributes to protection.

Some of the reasons for such conflicting results are that the investigators have used different antigenic preparations of Candida for immunization and different species of animals as hosts. The fact that different fungal antigens produce different responses in different hosts make it difficult to obtain a reproducible result or to make valid comparisons of the reports from different investigators. Candida albicans is a complex organism with numerous antigenic components. Its encounter with the host can produce a multitude of antigen and antibody interaction. Treatment of the organism chemically or physically by heat or disruption could readily alter or eliminate some of the antigenic components. Such modified antigens could be expected to produce a response which would differ from the one produced by living organisms.

Axelsen (1971) showed that antigens of C. albicans treated in different ways produced different numbers of antibodies in hyperimmunized rabbit antiserum as determined by cross-electrophoresis. He showed that Candida killed and partly disintegrated in 2% phenol produced 46 precipitates while homogenised antigen produced 68 precipitates.

In this study different antigens prepared from C. albicans produced different degrees of protection in mice as judged by survival times and rates after challenge with a lethal dose. CAD-6 antigen, which is a crude whole cell homogenate, proved the most

protective antigen in the system adopted. Next in the series was mannan on latex. The protective quality of this antigen and the immunity it conferred is attributed to the fact that it was recognized and became engulfed by macrophages. Perhaps the quantity adsorbed onto latex (viz. 25% of the total mannan present in the soluble preparation) could have been a more appropriate concentration for the elicitation of immune responses. The heat killed blastospores did not confer any resistance at all.

In the past, most investigators have used whole cells of Candida in the yeast phase, killed either by heat or chemical treatment. The criterion for acquired immunity has usually been the level of demonstrable agglutinin titres. Agglutinins are produced in response to antigen present in the outer cell wall of the organism. Frisk (1977) reported that Candida cells have a limited assortment of surface antigens and that mannan-protein determinants dominate. Antibodies to surface antigens can be produced even after a moderate fungal growth. Winner (1955) questioned the value of agglutinins because of their equal prevalence and titre in healthy and infected individuals. Their presence merely shows an experience with Candida antigens and this could be obtained from the organism in its commensal state, not as the result of an invasive process. Hellwege et al. (1972) found that agglutinating antibodies resulting from simple Candida colonization are mainly Igm whereas infections causing a rise in agglutinin titres induce predominantly IgG antibodies. On the basis of the data presented, it is possible that agglutinins produced by whole cells are directed towards the blastospore phase of the

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organism while the invading organism, by its ability to metamorphose to the mycelial phase, could evade the immune response to blastospores. Chattaway et al. (1968, 1976) found that yeast and mycelial phases differed chemically as well as physically. There was a difference in amino acid composition of proteins with presence of histidine but no arginine in the blastospore fraction and the reverse of this distribution in the mycelial fraction. The mycelial phase had 3 times more chitin with a cell wall approximately half as thick as the blastospore wall.

Denning and Davies (1973) determining the chemotaxis of polymorphonuclear leukocytes (PMN) by mannan found that suspensions of blastospores attracted significantly larger numbers of PMN than did a mycelial suspension (a ratio of 5:3) of similar weight or opacity. Reduced chemotactic activity is related to greater virulence (Cutler, 1977). Evans et al. (1973) found qualitative differences in antibodies produced by antigens from the two phases of C. albicans as determined by 2-dimensional immunoelectrophoresis (2D-IE) and double diffusion. Syverson et al. (1975) using 2D-IE found that the soluble cytoplasmic extract from both growth phases of C. albicans had at least 6 distinct antigenic constituents in addition to those which were shared.

Theoretically it is possible that when an animal is immunized with heat killed organism, the agglutinins are directed towards the modified surface antigen of the yeast phase. Although some yeasts are subsequently broken down and degraded in the body, exposing the inner antigens, this process could occur too late and the host's immunocompetent cells are primed and committed to the surface antigen.

However, such agglutinins may not be able to cope with antigens associated with the invading organism. This is considered to be the case in the present studies. Although mice vaccinated with heat killed cells produced higher titres of agglutinin than were elicited by other antigens they were not able to alter the course of local and systemic infections. Thigh muscle lesions in immunized mice ran the same course as in the control animals. Histopathology of the lesions showed the same type of cellular responses and the same duration of infection. The same end result viz lack of protection was noticed in systemic infections of all mice. Immunized animals died at the same rate as controls.

Kashkin (1975) reported that heated vaccine was less effective in inducing an immunological response in rabbits than soluble extracts with high protein concentrations. Giger et al. (1978) found that cutaneous lesions with viable C. albicans conferred protection to mice while heat killed was less effective. Lack of effectiveness of heat killed blastospores may be due to several causes, including:

1. The protective antigen may be heat-labile.
2. The protective antigen may not be located on the outer cell wall.
3. The protective antigen may become active only when the live parasite encounters the host.
4. The heat killed antigen may produce non-specific and pathogenically irrelevant antibodies.

If agglutinins have no protective qualities they may eventually hinder the immune system by binding with the organisms without eliminating them, by protecting them from phagocytosis and by blocking the proper antigenic stimulation. Windhorst and Stoltzner (1975) suggested that a situation could exist in candidiasis where the individual lacks the capacity to respond to an antigen of Candida which is of prime importance in pathogenesis. Such an individual could overcompensate and in doing so fail to control the infection by making non-significant antibody. Suppression of cell mediated immunity and high agglutinin titres has been reported in cases of mucocutaneous candidiasis by Takeya et al. (1976). Matsumoto (1976) reported 6 cases of chronic mucocutaneous having a dissociation of humoral and cellular response with high titre of agglutinating antibodies and impaired delayed hypersensitivity (DH) to Candida and PPD antigens. Suppression and CMI and increased antibodies has been reported in other chronic diseases caused by bacteria and parasites (Turk, 1975). Inverse relationship between DH and humoral response has been reported in lepromatous leprosy with an increase of the antibody response (Axelsen et al., 1974a).

The possibility that protective antigen may be hidden in the inner cell wall layers may be suggested by the finding that CAD-6 antigen gave the highest degree of protection in challenged mice. CAD-6 antigen is not heated or purified. However, it is homogenized, making all the antigenic components available to the host's immune system. It would be of interest to determine if the homogenate of the mycelial phase of the organism when added to the homogenate of the yeast phase would improve its protective value.

In the thigh lesion experiment living Candida cells conferred more protection to mice than killed organisms as measured by duration of infection and the morphological state of the parasite in the host tissue. Mice infected and self healed on three occasions developed demonstrable local resistance. Thus on the 5th day after challenge the infection in immunized mice was aborted while in the control it was still progressing. The histopathology of the lesion showed that the fungus was in the yeast phase in the immunized animals with the mycelium form predominating in the controls. According to Blyth (1958) fungal morphology may be an indicator of the efficacy of local defense mechanism. Where filamentous forms occur, host response to colonization of fungi is minimal, whereas when the yeast form is dominant the host response is successfully curtailing tissue colonization by the pathogens.

The spleens of the animals immunized and then given multiple infections were enlarged compared to the controls. Histopathology of the spleen showed hyperplasia with prominence of numerous large mononucleated cells which were considered to be macrophages. As to the role of macrophages in Candida infection, Stanley and Hurley (1969) showed that although normal peritoneal macrophages of mice actively phagocytized Candida the organism readily grew intracellularly. They suggested that such cells might contribute to extension and dissemination of infection by acting as vectors. Bird and Sheagren (1970) found that mice infected with C. albicans exhibited enhanced phagocytosis as measured by the clearance of colloidal carbon from the blood stream. Production of accelerated carbon clearance required living organisms. Salvin and Cheng (1971)

found that macrophages from guinea pigs sensitized to C. albicans were more destructive than those from the normal animal and that this resulted in a more limited spread of infection throughout the tissues, thereby rendering the host more resistant. Winner (1972) studying the phagocytosis of C. albicans by mouse peritoneal macrophages found no difference between macrophages of normal and immunized animals. After one hour a large proportion of the yeast cells were inside the macrophages; after 2 hours most of the fungi had produced germ tubes and after 4 hours mycelial filaments were formed. However he found a difference when macrophages from infected mice were used instead of normal or immunized animals. Infection had increased the capacity of macrophages to retain Candida intracellularly and this prevented dissemination of the organism throughout the body of the host. He associated this activation of macrophages to the development of delayed hypersensitivity.

Some microorganisms such as Mycobacterium and Brucella are not killed by normal macrophages though they can be digested by activated ones. Activated macrophages with highly microbial properties contribute largely and sometimes exclusively to the mechanism of host resistance (Mackaness, 1969).

Lack of an effective protection conferred by live Candida in systemic infection of mice in the present studies was attributed to the fact that the animals had not recovered from their local infection, nor had they developed a protective level of acquired immunity. It was observed that it takes one month for animals to recover from a local lesion. These animals had been given live

organisms at the inoculation sites and the challenge inoculum possibly constituted an overload. A better protection might have been observed if the period between sequential sub-lethal infections had been prolonged.

Another factor which is important in response to an antigen is the nature of the host. It appears that mice are more suitable hosts for induction of acquired immunity than rabbits. Guinea pigs are innately more resistant to Candida infections. Winner (1960) stated that the apparent threshold dose for fatal infection in guinea pigs is 80,000 organisms per gm body weight compared with that of 20,000 per gm for mice and 2,000 per gm for rabbits.

Host responses differ among individuals of the same species. Axelsen et al. (1974b) studying the host response of 15 patients with chronic mucocutaneous candidiasis to 78 known antigenic components of C. albicans found a variation in the number of antibodies present ranging from 2 to 39 per serum. All patients had antibodies to antigen 78, a mannan-protein complex. Antibody profiles appeared to be primarily related to the patient's inherent ability to develop humoral immune responses rather than the nature of the test antigen.

In the present work, precipitin tests on sera of the mice surviving systemic infection did not show any precipitins to soluble mannan. Demonstrable antibodies were directed extensively towards protein antigens. Lack of responsiveness to soluble polysaccharides of different microorganisms have been reported in white mice. Felton (1949), Felton et al. (1955) and Kaplan et al. (1950) reported that type II and III pneumococcal polysaccharides injected to white mice

persisted in the tissues and did not appear to be metabolized there. As the result of persistence of antigen in the body there was either an interference with antibody production or the newly formed antibody was continuously neutralized. Kozel et al. (1977) reported the unresponsiveness of mice to cryptococcal polysaccharide with reduced ability to produce antibody. Immunofluorescent studies showed a long term deposition of polysaccharide in the tubular epithelial cells of the kidneys.

Mathews and Inman (1968) reported that the nature of antibody globulins produced by rabbits immunized with heat-killed Candida was mostly directed against mannan. In man, precipitins to both mannan and protein are reported in healthy and diseased subjects. Humoral responses to such a complex organism could be pathogenic, non-specific, interfering and sometimes protective depending on the nature and the dose of the antigen and immunological competence of the host. Soluble mannan diffusing out of the organism when it comes into contact with mucous membrane could act as an allergen in some individuals causing manifestations of vaginitis, pruritis ani, chronic urticaria and asthma (Holti, 1966; Pepys et al., 1968 and Stanley and Hurley, 1974). Precipitins to mannan have been demonstrated in such allergic cases. Colonization of Candida on a host as a commensal or the use of a whole cell vaccine in experimental animals produce agglutinins which are directed to the outer cell wall. They are not protective and have little diagnostic value. Although their quantities may serve as an index of antigen load they cannot correlate with degree of invasion of the host tissues. Protective antibodies are produced

in the host as a result of active participation of host and parasite as a result of localized lesions or systemic invasion. Such a protective antigen has not yet been identified. It could be in the inner cell wall which becomes exposed only after degradation or it could be a metabolite of living organisms whose activity is enhanced when in contact with host tissue. It cannot be a purified, soluble single protein or polysaccharide antigen since such preparations neither stimulated lymphocytes *in vitro* nor protected the immunized animals *in vivo* in the present studies. It is perhaps more likely to consist of a complex containing a larger proportion of protein moiety and traces of polysaccharide. Culture filtrate might theoretically act as a protective antigen except that the mannan content would generally be too high. Perhaps culture filtrate of the mycelial phase would be rather different. It would be difficult to identify the protective antigen although one indirect approach which might be helpful is its recognition by antibody production.

It would be of some help in establishing the identity of protective antigens if the surface mannan of the live *Candida* could be removed without altering or destroying the remaining antigen complex. Production of antibody to the homogenate of this preparation might give a clue to the hidden antigen.

Another potentially valuable study would be to compare the antibodies produced by heat-killed blastospores of *C. albicans* and those produced in animals surviving challenge with live organisms.

In the light of present knowledge resistance against C. albicans depends upon both cellular and humoral factors, reflecting the immune capacity of the host. Innate and acquired resistance blend in overall protection. All the immune mechanisms are indispensable for protecting the host from this opportunistic fungi. Each mechanism has a role and the entire immune system may have to work in concert.

The contribution made by CMI is likely to be the recognition of the antigen by the sensitized lymphocytes at the site of entry, aborting an infection through production of lymphokines. Pearsall et al. (1973) reported that mouse lymphocytes stimulated with PHA produced lymphokines which were toxic to Saccharomyces cerevisiae and Candida albicans. Rocklin (1974) described a soluble product, following stimulation of lymphocytes with specific antigen, called LIF (leukocyte inhibitory factor) which was distinct from MIF. He suggested that this product could produce a link between the lymphocytes and the inflammatory process. T cells also contribute indirectly by cooperating with B cells in production of certain types of antibodies.

Macrophages have an important role linking innate and acquired immune mechanisms. They ingest the particulate antigens and assist in its subsequent processing and presentation to T cells. They can eliminate microorganisms when activated. They also function in humoral reactions. Antibody-coated microorganisms encourage phagocytosis by macrophages and polymorphonuclear leukocytes in conjunction with complement. Antibody has been demonstrated on engulfed blastospores of C. albicans by leukocytes (Ishikawa et al. 1972).

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It has been suggested that salivary IgA antibody may limit the infection of the mucosa by C. albicans (Lehner et al. 1972). The contribution of protective antibody would be greatest when the infection becomes systemic.

Although much work has been done on the relationships between C. albicans and its host, the individual components of the immune system, their relationships to susceptibility and acquired resistance, and their integration with non-specific defence mechanisms remain largely unsolved. It is unlikely that either susceptibility or resistance will be linked to single factors or mechanisms of the immune response. Much additional critical investigation will be necessary before a clearer picture emerges of the interrelationships between this most ubiquitous of yeasts in man's internal environment, and the factors responsible for altering host-parasite relationships to man's disadvantage.

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