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Immunological Relatedness Among *Candida albicans* and Other Pathogenic *Candida* Species

R. F. HECTOR,* F. L. LYON, AND J. E. DOMER

Department of Microbiology and Immunology, Tulane University School of Medicine, New Orleans, Louisiana 70112

Received 13 July 1981/Accepted 19 August 1981

Membrane-mitochondrial (butanol-hot phosphate-buffered saline) and cytosol (soluble cytoplasmic substances) extracts from seven pathogenic species of *Candida* were used in in vivo and in vitro immunological assays to study antigenic similarities among the strains with respect to *C. albicans*. Mice were sensitized with *C. albicans* serotype A for footpad testing or to provide cells for lymphocyte stimulation assays, and guinea pigs were immunized with whole cells or butanol-hot phosphate-buffered saline extracts of *C. albicans* to obtain antisera for immunodiffusion assays. When extracts from each of the seven species were used in the assays, they consistently segregated, as determined by statistical or subjective analyses, into three groups. Extracts of *C. albicans* serotype A or B and *C. stellatoidea* were the most immunologically reactive in all assays, indicating close similarities between those two species, whereas extracts of *C. tropicalis* and *C. parapsilosis* elicited only moderate responses. Extracts from *C. krusei*, *C. guilliermondii*, and *C. pseudotropicalis* were hypo- or nonreactive in the assays, indicating a low level of antigenic relatedness to *C. albicans*.

Candida albicans and, to a lesser extent, other species of *Candida* are found frequently as a part of the normal flora of humans. When the normal physiological or immune state of the individual is altered, e.g., by diabetes, pregnancy, or immunosuppressive therapy, chronic superficial or severe systemic disease may follow. Of the 81 species of *Candida* recognized by Lodder (13), only 7 are known to be regularly pathogenic, i.e., *C. albicans*, *C. stellatoidea*, *C. tropicalis*, *C. parapsilosis*, *C. pseudotropicalis*, *C. guilliermondii*, and *C. krusei*. Although there have been a number of reports dealing with the relatedness of these species, the results seem to depend on the methodologies employed. Thus, the associations determined by agglutination or precipitation of cell wall antigens (5, 10, 23) are not necessarily the same as those determined by studies of cytoplasmic antigens (15) or deoxyribonucleic acid homologies (12, 22).

One of the interests of this laboratory in the past few years has been the demonstration of immune responses in experimental candidiasis and the development of suitable antigens to detect certain of those responses. Two antigenic preparations which have been found to be useful in our studies are soluble cytoplasmic substances (SCS) and a butanol and hot phosphate-buffered saline extract (B-HEX) of the membrane-mitochondrial fraction of disrupted blastospores (4, 16). The purpose of the present study was to investigate the relatedness of the major pathogenic species of *Candida* by using B-HEX and SCS from each in in vivo and in vitro assays. When the extracts were used in footpad assays in mice sensitized to *C. albicans*, in lymphocyte stimulation assays (LSA) using cells from similarly sensitized mice, and in Ouchterlony double-diffusion tests against antisera to whole cells of *C. albicans* or *C. albicans* B-HEX, the results allowed us to categorize the strains tested into three groups based on their degree of similarity to a single strain of *C. albicans* serotype A. In addition, the extracts have shown their potential value as antigens for the detection of delayed-type hypersensitivity and antibody and in eliciting a blastogenic response in an animal model for candidiasis.

MATERIALS AND METHODS

Source and culture of organisms. The strains of *Candida* used for the various experiments were as follows: *C. albicans* B311 (serotype A) from H. Hasenclever; *C. albicans* 20A (serotype A), *C. albicans* B1012a (serotype B), and *C. albicans* 526B (serotype B), all obtained from E. Reiss, Centers for Disease Control; *C. stellatoidea* 44 and C, *C. tropicalis* 53 and 1525, *C. pseudotropicalis* CDC-148 and Martin, *C. krusei* 351 and 2900, *C. guilliermondii* 2484 and 2309, and *C. parapsilosis* CDC-60, all from the Tulane collection; and *C. parapsilosis* 20246 from the American Type Culture Collection. All strains were confirmed as to species by morphology, i.e., presence of

pseudohyphae and blastospores, plus or minus chlamydospores, and by sugar assimilation tests. Stock cultures of all strains were maintained by monthly transfers on modified Sabouraud dextrose agar slants (Scott Laboratories, Inc., Fiskville, R.I.) and were stored at 4°C.

To prepare subcellular components from each strain, washed blastospores suspended in tris-(hydroxymethyl)aminomethane buffer were disrupted by ballistic action in a Braun homogenizer, and the homogenate was separated into cell walls, membranes-mitochondria, and soluble cytoplasmic substances (SCS) as described previously (4). The membrane-mitochondria fraction was dialyzed against distilled water, lyophilized, and then treated with 1-butanol according to the method of Cohen and Warringa (2). The membrane-mitochondria–butanol mixture (4 mg of membrane-mitochondria fraction per ml of 1-butanol) was held in an ice bath for 20 min with continuous stirring and then allowed to stand undisturbed for an additional 10 min before centrifugation at 400 × *g*. The supernatant was discarded, and the pellet was dried under a stream of N_2, stored in a desiccator overnight to remove traces of butanol, and extracted with phosphate-buffered saline (pH 7.4) for 1 h at 50°C (16). Proteins were precipitated from the saline extract with ammonium sulfate (100% saturation), and the precipitate was redissolved in nonpyrogenic saline before dialysis against the same solvent. Protein content was determined by the method of Lowry et al. (14), using bovine serum albumin as a standard. The extract was then diluted to the appropriate concentration and stored at −20°C until used. The butanol–heat-extracted precipitated preparations will hereafter be referred to as B-HEX. The SCS was dialyzed against distilled water, lyophilized, and stored in vacuo over desiccant.

Animal sensitization. Male CBA/J mice (Jackson Laboratories, Bar Harbor, Maine), approximately 10 to 12 weeks old at the beginning of the experiments, were used throughout these studies. Mice were sensitized as described previously (6) by two intracutaneous inoculations of 10^6 *C. albicans* B311 blastospores suspended in 0.05 ml of nonpyrogenic saline, given 14 days apart.

Antisera were collected from Hartley male guinea pigs, 250 to 300 g, inoculated initially with either 2 × 10^8 viable *C. albicans* B311 blastospores emulsified in Freund complete adjuvant or with 500 μg of *C. albicans* B311 B-HEX protein. All animals were given two booster inoculations 1 month apart with cells or B-HEX emulsified in Freund complete adjuvant. Periodic bleedings were taken before and after booster inoculations, and those sera resulting in the strongest precipitin reactions when tested with the appropriate antigen, i.e., either SCS or B-HEX derived from *C. albicans* B311, were then used in the immunodiffusion assays.

Lymphocyte stimulation. Seven days after the second inoculation of viable *Candida* cells, inguinal lymph nodes draining the site of cutaneous inoculation of three infected mice, and the corresponding nodes in three uninfected mice, were excised and pooled in their respective groups. Single-cell suspensions were prepared by teasing the nodes apart in cold RPMI 1640 medium containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 20 mM L-glutamine, 100 U of penicillin and 100 μg of streptomycin per ml, 5% (vol/vol) heat-inactivated normal pooled horse serum (GIBCO), and 5×10^{-5} M 2-mercaptoethanol (1) (Calbiochem, La Jolla, Calif.). After filtering through sterile gauze, the cells were washed in complete medium and suspended to a concentration of 2.5×10^6 viable cells per ml, as determined by hemacytometer counts of cells stained with trypan blue (GIBCO). Microcultures were set up in flat-bottom microtiter trays (Microtest II; Falcon Plastics, Oxnard, Calif.) by delivering 0.1 ml of cells (2.5×10^5 per well) and 0.1 ml of complete medium alone or complete medium containing the appropriate concentrations of antigen or mitogen to each well. The mitogens used were purified phytohemagglutinin (Wellcome Reagents, Ltd., Beckenham, England), 0.05 μg per well, and *Salmonella typhosa* O901 lipopolysaccharide B (Difco Laboratories, Detroit, Mich), 50 μg per well. The antigens used were B-HEX extracts from all strains listed above. All conditions were prepared in triplicate, and trays were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Eighteen hours before harvesting with a MASH II unit (Microbiological Associates, Bethesda, Md.), each well was pulsed with 1 μCi of [*methyl*-^{3}H]thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) delivered in 10 μl of nonpyrogenic saline. Mitogens were harvested on day 4 of incubation. After drying at 100°C for 20 min the glass fiber disks were punched out into plastic liquid scintillation vials (New England Nuclear Corp.), and 10 ml of liquid scintillation solution containing 3.66 g of 2,5-diphenyloxazole (PPO; New England Nuclear Corp.) and 0.07 g of *p*-bis-(*O*-methylstyryl)-benzene (New England Nuclear Corp.) per liter of scintillation-grade toluene (J. T. Baker Chemical Co., Phillipsburg, N.J.) was added. Samples were counted in a Beckman LS250 for a minimum of 10 min or 10,000 counts, and the results were expressed as the mean of the three samples in counts per minute.

Footpad testing. The B-HEX antigens used in the footpad assay were from the following strains: *C. albicans* B311, 20A, and 526B; *C. stellatoidea* 44; *C. tropicalis* 53; *C. krusei* 351; *C. guilliermondii* 2484; *C. parapsilosis* 20246; and *C. pseudotropicalis* CDC-148. Antigens were administered singly to mice 6 days after the second cutaneous inoculation of *C. albicans* B311. Uninfected mice were used as controls. Footpad testing was accomplished by the injection, with a micrometer syringe, of 0.02 ml of test preparation, 20 μg protein per ml, suspended in nonpyrogenic saline. Each foot was measured with a Schnelltäster calipers (H. Kröplein GmbH, Schluchtern, West Germany) before and at 15 min and 4, 7, 24, and 48 h after injection. The mean net increase in footpad thickness for each group was determined after the subtraction of pre- and post-inoculation values. In each experiment the animals were randomized, coded, and injected by one individual and then measured by a second individual to whom the code was unknown.

Immunodiffusion. The antigens and antisera were tested for precipitin reactions by a double-diffusion

(Ouchterlony) technique using commercial immunodiffusion plates (pattern B; Hyland Diagnostics, Deerfield, Ill.). After comparisons of all strains had been made, the agar in fresh plates was melted and repoured, to allow for the preparation of a pattern of wells different from those precut, and used in further analyses prepared specifically for photography. SCS preparations were dissolved in phosphate-buffered saline (pH 7.4), 5 mg/ml, and the B-HEX preparations ranged from 2.0 to 3.5 mg of protein per ml.

Statistical analyses. When indicated, data were analyzed by one-way analysis of variance followed by Tuckey's w procedure for the comparison of means. Significance was determined at the $P = 0.05$ level.

RESULTS

Lymphocyte stimulation with B-HEX antigens. Membrane extracts prepared from the 16 strains of *Candida* were tested for their ability to stimulate in vitro lymphocytes from mice infected with viable *C. albicans* B311 blastospores and from uninfected controls. Most extracts were tested at concentrations of 10, 20, and 40 μg of protein per test well, based on results of previous experiments with *C. albicans* B311 extracts. Phytohemagglutinin and lipopolysaccharide were included as mitogen controls. The responses to the mitogens were within normal ranges for the dose used, and no specific data for them are presented. Results of the LSA using the *Candida* antigens are summarized in Fig. 1. The optimal concentration of B-HEX antigen varied from preparation to preparation, and the results presented are those for the concentrations showing the maximum stimulation. Moreover, the data presented here are from a single experiment, but similar results were obtained in two subsequent experiments using a similar protocol. Statistical comparisons are presented for infected groups only, since there were minor differences only among control values.

As can be seen in Fig. 1, the extracts varied widely in their ability to stimulate lymphocytes from mice infected with *C. albicans* B311. The extracts could be grouped roughly into three categories, however, representing low, moderate, and strong stimulatory capacities. Those extracts in the strong category, and therefore capable of stimulating lymphocytes to the same

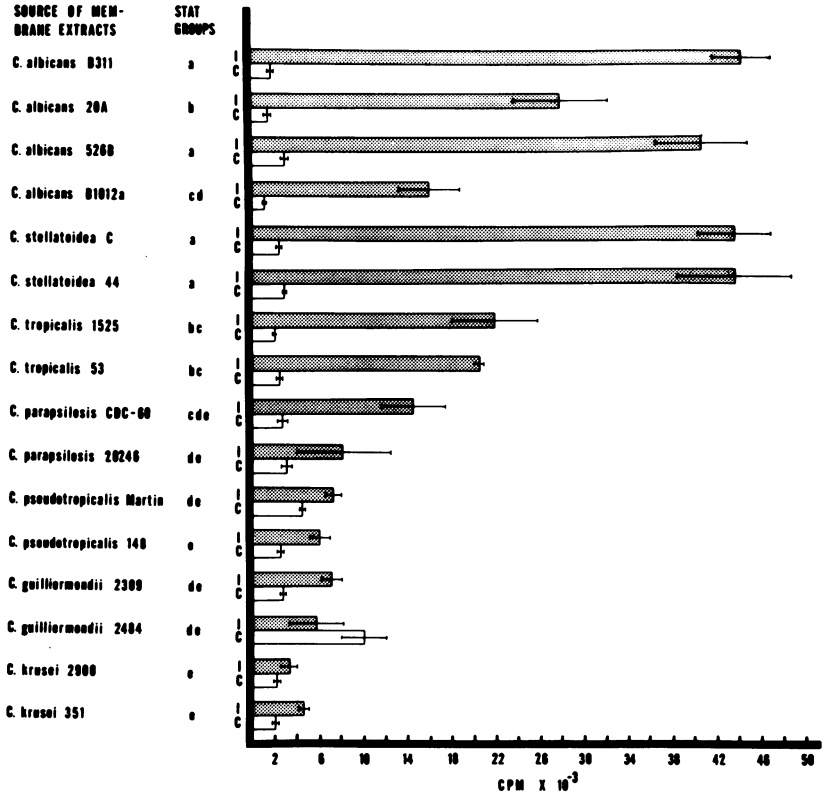

FIG. 1. *LSA using B-HEX extracts with lymph node lymphocytes from uninfected mice (C) or mice infected (I) with C. albicans B311. The means of the infected groups have been ranked statistically by Tuckey's w procedure ("stat groups"). Those extracts followed by the same letter are not statistically different (P = 0.05).*

high degree as the homologous extract, included one strain of *C. albicans*, 526B (a serotype B strain), and both strains of *C. stellatoidea*.

Both strains of *C. pseudotropicalis*, *C. guilliermondii*, and *C. krusei* and one strain of *C. parapsilosis* (20246) stimulated the sensitized lymphocytes to only low or insignificant levels of response. In the two subsequent experiments, however, strain 20246 B-HEX elicited responses which fell into the moderate category. One extract of *C. guilliermondii*, for reasons unknown, was stimulatory to control cells.

B-HEX preparations resulting in intermediate levels of stimulation were derived primarily from *C. tropicalis* and *C. parapsilosis* strains, as well as two of the *C. albicans* strains, 20A and B1012a. The latter two strains are representatives of serotypes A and B, respectively, which precluded any correlation between the degree of stimulation and the serotype used.

Statistical analyses of the results supported the separation of the strains into the above three groups, and these are included in Fig. 1. Those organisms with the same letter designation under the heading "stat groups" have no statistical differences.

Footpad testing of extracts. B-HEX preparations from three strains of *C. albicans*, two serotype A and one serotype B, and from a single representative, chosen at random, of each of the other six *Candida* species used in this study were tested for their ability to detect delayed-type hypersensitivity in mice infected cutaneously with viable blastospores of *C. albicans* B311. Uninfected mice were used as controls. The results of the footpad assays, including statistical comparisons, are presented in Fig. 2. The data presented are from a single experiment, but similar results were obtained from a subsequent experiment using a similar protocol. Although the footpads were measured at five intervals after the injection of the various B-HEX preparations, the increase in footpad thickness was maximum at 24 h, and only those readings are presented for comparison. All of the cutaneously infected groups displayed immediate-type hypersensitivity reactions at 15 min, but those reactions had greatly diminished in all animals by 3 h. Although the delayed component of the reaction was maximum by 24 h, the footpads of infected animals still had substantial swellings at 48 h.

As was seen in the LSA, there was a broad range of reactions with the extracts employed. The three *C. albicans* extracts and the *C. stellatoidea* B-HEX elicited the greatest responses, none of which was significantly different from the others on a statistical basis ($P = 0.05$). The extracts of *C. guilliermondii*, *C. pseudotropicalis*, and *C. krusei* elicited the least response in

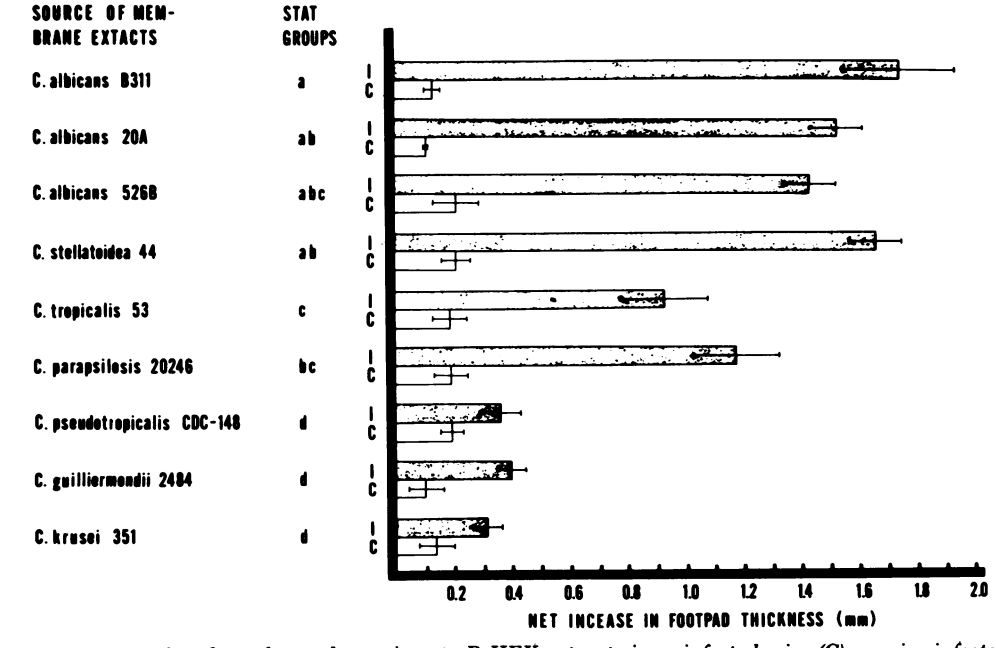

FIG. 2. *Twenty-four-hour footpad reactions to B-HEX extracts in uninfected mice (C) or mice infected (I) with C. albicans B311. The means of the infected groups have been ranked statistically by Tuckey's w procedure ("stat groups"). Those extracts followed by the same letter are not statistically different (P = 0.05).*

footpads of mice infected with *C. albicans*, and those of *C. parapsilosis* and *C. tropicalis* elicited reactions of intermediate intensity. None of the extracts elicited substantial reactions in unsensitized animals. Again, the statistical analyses, shown in the same figure, support the basic division of the seven species into groups of low, intermediate, and strong reactivity.

Immunodiffusion. B-HEX and SCS extracts from all 16 strains employed in this study were used in Ouchterlony double-diffusion assays against antisera raised in guinea pigs either to *C. albicans* B311 blastospores or to B-HEX derived from the same strain. Examples of some of the precipitin lines can be seen in Fig. 3a and 4a, photographs of stained slides prepared specifically for photography. Since a few of the weaker reactions are not discernible in the photograph, but were seen clearly in earlier assays, composite drawings of the reactions have been prepared to illustrate them (Fig. 3b and 4b). Furthermore, since no differences could be detected among all strains of *C. albicans* tested, whether serotype A or B, nor between strains within a given species, a single representative of each species was selected for photography and the composite drawings.

Despite the fact that double immunodiffusion as used here is a relatively crude method for demonstrating the total number of antigen-antibody reactions occurring between reactants, it is apparent from the photography and drawings that the same species that grouped together in the LSA and footpad assay also grouped together in this assay. Thus, regardless of the antigen or antiserum used, *C. albicans* and *C. stellatoidea* reacted completely with lines of identity, and *C. tropicalis* and *C. parapsilosis*,

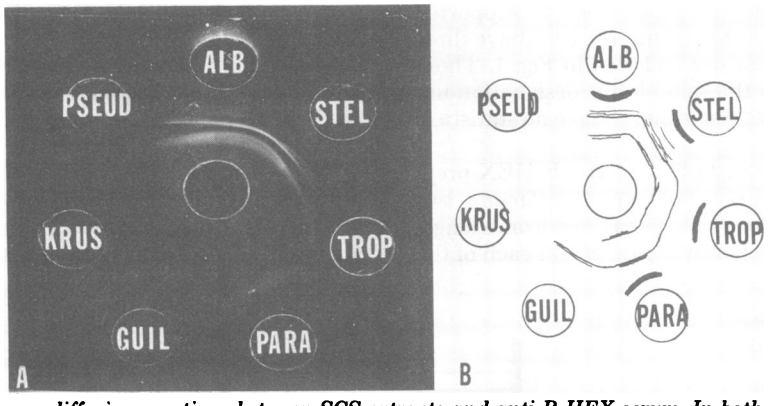

FIG. 3. *Immunodiffusion reactions between SCS extracts and anti-B-HEX serum. In both the photograph (A) and the composite (B) the SCS extracts from the seven species indicated were placed in the outer wells, while the anti-B-HEX was placed in the center well. The composite is a summary of preliminary assays which showed several precipitin lines not evident in the photograph.*

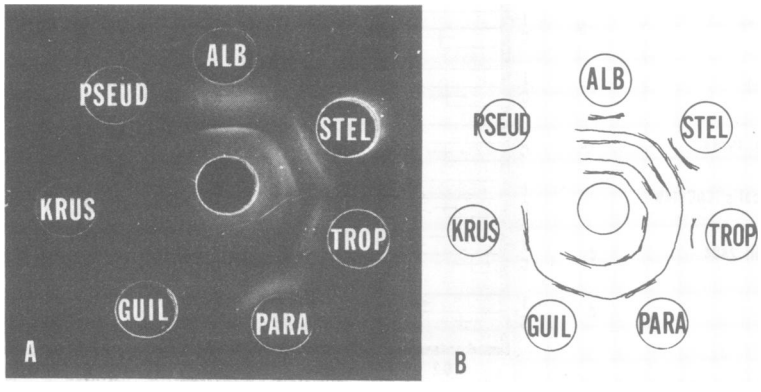

FIG. 4. *Immunodiffusion reactions between B-HEX extracts and anti-B-HEX serum. In both the photograph (A) and the composite drawing (B) the B-HEX preparations from the seven species indicated were placed in the outer wells, while the anti-B-HEX was placed in the center well. The composite is a summary of preliminary assays which showed several precipitin lines not evident in the photograph.*

while having fewer precipitin lines, still shared identity with the first group. The extracts of *C. guilliermondii*, *C. krusei*, and *C. pseudotropicalis* were weakly or nonreactive with either of the antisera, and some of the reactivity seen in earlier assays was lost due to deterioration of either the antigen or the antibody during storage, so that precipitin lines could not be demonstrated at the time the photographs were taken.

The precipitin patterns of the SCS and B-HEX extracts with the antisera to whole cells are not shown because they basically demonstrate the same relationships observed with the antisera to B-HEX. For example, the SCS preparations from *C. albicans* and *C. stellatoidea* each had a minimum of five precipitin lines, all showing identity, whereas the three precipitin lines from the SCS of *C. tropicalis* also had identity with the lines of the first group. SCS preparations from *C. parapsilosis* and *C. guilliermondii* each developed only two precipitin reactions showing identity with each other and to two of the three *C. tropicalis* precipitin lines. Additionally, *C. krusei* and *C. pseudotropicalis* each had a single precipitin line which had identity with a line from each of the other species, but not with each other. Finally, all of the antigens reactive in the B-HEX extracts for each species could be found also in the SCS extracts, but the SCS extracts had additional reactants in all but those from *C. parapsilosis*, *C. krusei*, and *C. pseudotropicalis*.

DISCUSSION

We have shown, based on the results of the LSA, footpad assay, and immunodiffusion assays, that the seven pathogenic species of *Candida* used in this study can be divided into three groups based on the antigenic similarities of membrane (B-HEX) and cytoplasmic (SCS) extracts, with respect to their relatedness to *C. albicans* B311. The extracts from strains of *C. albicans* and *C. stellatoidea* comprised the most reactive group, *C. tropicalis* and *C. parapsilosis* were consistently moderate in reactivity, and strains of *C. guilliermondii*, *C. krusei*, and *C. pseudotropicalis* were hypo- or unreactive in the assays.

In all three assays, the extracts of *C. stellatoidea* strains gave, where applicable, results which were statistically indistinguishable from those of the homologous *C. albicans* B311 extracts. In the immunodiffusion assays, there was usually complete identity seen in the precipitin lines formed between the two species and the antisera. There were no differences in the results of the LSA and footpad assays for strains of *C. albicans* which could be correlated with differences in serotype. Specifically, B-HEX from strain 526B (serotype B) caused stimulation of lymphocytes derived from mice immunized with strain B311 (serotype A) to a degree which was not significantly different from the homologous B311 extract, whereas the extracts of strain 20A (serotype A) and B1012a (serotype B) were somewhat less reactive. In the footpad assay, however, B-HEX from strain 20A was as reactive as the homologous B311 extract, on a statistical basis. In the moderately reactive group, extracts from *C. tropicalis* were consistently more reactive than those from *C. parapsilosis* in each of the assays.

It is difficult to correlate our results with those found in the numerous studies published dealing with the relatedness of the clinically important species of *Candida*, because in the majority of these reports interactions of antisera and cell wall antigens in agglutination or precipitin reactions were studied. In several of these reports a close relationship was demonstrated between *C. albicans* serotype A and *C. tropicalis*, whereas serotype B was shown to be antigenically similar to *C. stellatoidea* (5, 10, 23). Workers using different methodologies, however, obtained results conflicting with those mentioned above. When cytoplasmic antigens were used instead of cell wall antigens, little relationship could be demonstrated between *C. tropicalis* and *C. albicans* (15). Additionally, deoxyribonucleic acid homology studies (12, 22) showed that whereas *C. albicans* and *C. stellatoidea* were closely related, there was little homology between *C. albicans* and *C. tropicalis*. Thus, it is difficult to draw conclusions from the published reports due to the fundamental differences between methodologies and antigens employed.

Although the strains of *C. krusei*, *C. guilliermondii*, and *C. pseudotropicalis* fell into the least reactive group, it should be emphasized that they are not necessarily closely related to each other. In the immunodiffusion assays, *C. guilliermondii* and *C. krusei* shared a precipitin line, as did *C. krusei* and *C. pseudotropicalis*, but there is no direct evidence of the magnitude of similarities among these strains since our comparisons are relative to *C. albicans* B311. In fact, in immunofluorescence studies of several species of *Candida* using antisera raised in rabbits, it was found that homologous antigens must be used for the detection of infections due to *C. krusei*, *C. pseudotropicalis*, and *C. guilliermondii* (19). Here again, however, the author was looking at surface antigens. It is felt that the main antigens on the *Candida* cell surface are most likely comprised of mannan (9), while in our studies the antigens, especially the B-HEX

precipitated with ammonium sulfate, are most likely protein or glycoprotein in nature.

Though there have been numerous reports on the subject (3, 6, 17, 18, 21), it is not yet clear whether cell-mediated or antibody-mediated immunity is dominant in fighting infections due to *Candida*. Although there have been several reports on the value of serologies in detecting candidiasis (7, 8, 11, 20, 24, 25), little attention has been given to the detection of delayed-type hypersensitivity. It is clear from this and previous studies from this laboratory that the B-HEX antigen can be used successfully in the murine animal model for the detection of delayed-type hypersensitivity in infected animals. What has not been determined is whether immune responses can be detected with B-HEX from *C. albicans* in animals sensitized with any of the six other pathogenic species of *Candida*, and whether there is any cross-reactivity with any of the agents capable of causing systemic mycoses. Such studies are currently being planned.

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