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Demonstration and Solubilization of Antigens Expressed Primarily on the Surfaces of *Candida albicans* Germ Tubes

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Antisera against mycelial-phase, but not yeast-phase, *Candida albicans* absorbed with yeast-phase organisms preferentially stained germ tube segments of several strains of mycelial-phase *C. albicans* by the indirect fluorescent-antibody staining technique. Germ tube segment antigens were not found in significant amounts on blastospore segments or on yeast-phase organisms. Absorption of the mycelial-phase reference sera with yeast-phase *C. stellatoidea*, but not with *C. tropicalis*, *C. guillermondii*, or *Saccharomyces cerevesiae*, resulted in preferential germ tube segment staining of *C. albicans*. A dithiothreitol extract of mycelial-phase *C. albicans* organisms blocked staining of the germ tube segment, but a dithiothreitol extract of yeast-phase organisms did not. When dithiothreitol extracts from both phases were reacted against yeast-absorbed reference sera in tandem crossed and crossed line immunoelectrophoresis, a cross-reacting arc and several arcs unique to the mycelial-phase extract were noted. Immunofluorescent staining tests were performed, using appropriately absorbed sera from patients with candidiasis to stain a laboratory strain of *C. albicans*. Human tissue slices infected with *C. albicans* were used as targets for appropriately absorbed rabbit antisera. These human data indicated that antigens preferentially expressed on the germ tube in vitro were also expressed on filamentous structures of the fungus in infected human tissues. In vitro and in vivo, the invasive mycelial phase of *C. albicans* expresses certain antigens that are highly concentrated on the germ tube.

Infections by *Candida albicans* and other species of *Candida* occur frequently in immunocompromised patients (32). Manifestations of visceral candidiasis are frequently subtle and blood cultures are often negative (40). When blood cultures are positive, distinguishing transient fungemia (such as that seen with intravenous catheters) from systemic disease can be difficult (9). To provide a noninvasive aid to diagnosis, many serological tests have been developed, but as it is difficult to grow large quantities of mycelial-phase *C. albicans* organisms (29), nearly all serological tests have detected antibodies against antigens isolated from yeast-phase organisms or antigens present in large amounts in yeast-phase organisms (5, 6, 12, 14, 19, 21, 22, 31, 35, 40, 44). Because yeast-phase *C. albicans* normally colonizes skin folds, mucous membranes, and the gastrointestinal tract, antibodies to yeast-phase cell wall mannan (CWM) are ubiquitous, even in healthy individuals (5, 12, 21, 31, 40, 44). This has contributed to the difficulty in distinguishing colonization from invasive disease. During tissue invasion, mycelial-phase *C. albicans* is virtually always found (32, 45). Detection of antigens unique to the mycelial phase might provide a specific indication of tissue invasion (10, 11, 29, 32).

By using crossed immunoelectrophoresis or double immunodiffusion, several groups have reported finding cytoplasmic antigens unique to each phase of single strains of *C. albicans* (11, 19, 39). Using O'Farrell two-dimensional electrophoresis, Manning and Mitchell also found cytoplasmic proteins and antigens unique to the mycelial phase of one strain but noted that all of those were present in yeast-phase cytoplasmic extracts from another strain (28, 30).

Conflicting results have emerged from comparative studies of *Candida* surface antigens. One group could not detect a significant difference in staining of the two forms with ferritin-conjugated antibodies raised against nonviable yeasts (42); others reported more intense staining of the germ tube segment than of the blastospore segment after exposure to highly dilute sera from patients with candidiasis (16, 20). Both groups speculated that antigens unique to the germ tube segment account for this.

By the use of immunofluorescent staining and quantitative immunoelectrophoresis, we compared yeast-phase and mycelial-phase surface antigens from multiple strains of *C. albicans*, as well as several related species of yeast. Evidence for antigens highly concentrated on the germ tube segment of multiple strains was developed. Data are presented indicating that these antigens are expressed on the surface of germ tubes of *Candida* spp. invading human tissues and that patients with invasive candidiasis are stimulated to produce antibodies against germ tube antigens.

MATERIALS AND METHODS

Source and culture of organisms. *C. albicans* type A B311 (kindly provided by Edward Balish, University of Wisconsin, Madison) was used for most experiments. *C. albicans* type B 792 (a gift from John Bennett, National Institutes of Health, Bethesda, Md.) and fresh clinical isolates of *C. albicans* from patients at our medical center were used in several experiments. Identity of the clinical isolates was confirmed in our clinical laboratory by testing for germ tube formation and growth of chlamydospores on appropriate media. Cultures of *Candida guillermondii*, *Candida stellatoidea*, *Candida tropicalis*, and *Saccharomyces cerevisiae* were obtained from the Wisconsin State Laboratory of Hygiene.

A slight modification of Lee media (26) was inoculated with 2×10^6 to 5.0×10^6 organisms per ml and rotated at 150 rpm at 22 to 25°C for 42 to 48 h. If conversion to mycelial phase was desired, the final yeast suspension was diluted

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into 20 volumes of modified Lee media prewarmed to 37°C and agitated for 4 to 6 h at 150 rpm. Under these conditions, 89 to 98% of B311 organisms produced germ tubes. Germ tube forms of *C. stellatoidea* were obtained by continuing the 37°C incubation for 24 h. The cells were harvested by passing through a fritted glass filter or by centrifugation and then were washed twice with phosphate-buffered saline (PBS; pH 7.2). If desired, the organisms were killed by suspending them in 10% phosphate-buffered Formalin overnight at 4°C. Cells were washed three times with sterile PBS and suspended in sterile PBS for storage at 4°C.

Preparation of antigens. Cytoplasmic extracts were prepared as previously described (21). Lyophilized samples from the yeast- and mycelial-phase organisms were labeled yeast cytoplasmic extract (YCYE) and mycelial cytoplasmic extract (MCYE), respectively. CWM was produced by the method of Peat et al. (33) as modified by Sakaguchi et al. (36).

A modification of the method of Chattaway et al. (4) was used to extract surface components. Wet, pelleted organisms were suspended in 10 volumes of 0.05 M Tris-hydrochloride (pH 7.5) containing 0.8 M mannitol. Dithiothreitol (DTT) (0.5 M) in Tris-mannitol buffer was added to a final concentration of 12 mM DTT, and the solution was incubated under N_2 in a sealed vessel at 37°C in a shaking water bath. After 4 h, 0.5 M iodacetamide in Tris-mannitol buffer was added to a final concentration of 17 mM iodacetamide, and the solution was stirred at room temperature for 2 h. The suspension was centrifuged at 600 × *g* for 10 min, and the supernatant was dialyzed for 48 h against deionized distilled water in nitrogen-purged dialysis tubing with a 3,500-molecular-weight exclusion (Spectrum Medical Industries Inc., Los Angeles, Calif.).

For KOH extraction, yeast-phase or mycelial-phase forms were first disrupted in a French press and centrifuged as described above. The cell walls were agitated in chloroform-ether (1:1 [vol/vol]) for 4 h, the solvent was aspirated, and the chloroform-ether extraction was repeated for 24 h at room temperature. Solvent was aspirated, and the cell walls were suspended in chloroform for 24 h, followed by solvent aspiration and air drying overnight. These defatted cell walls were suspended in distilled water for collection and lyophilized. The dried, defatted cell walls were suspended in 1 N KOH in screw-capped plastic bottles, flushed with N_2, sealed, and agitated for 60 min at 30°C in a shaking water bath. The solution was then centrifuged at 600 × *g* for 10 min at 0°C, and the supernatant was placed in dialysis tubing with a 3,500-molecular-weight cutoff. The tubing was purged with N_2 and sealed, and exhaustive dialysis against distilled water was performed at 4°C for 96 h. The dialyzed extracts were filtered through a membrane prefilter (Millipore Corp., Bedford, Mass.) before being lyophilized and stored at −20°C.

Immunizations. Unless otherwise noted, intact organisms of or extracts from *C. albicans* B311 were used for all injections. Immune sera to intact organisms were produced by intramuscular injections of either Formalin-killed mycelial-phase organisms (FKM) or Formalin-killed yeast-phase organisms (FKY). New Zealand white rabbits were injected with 5×10^8 organisms in 0.5 ml of complete Freund adjuvant biweekly for 7 to 10 injections and then monthly or bimonthly. Antisera specific against cytoplasmic extracts were produced by an initial intramuscular injection of 3.0 mg of YCYE or MCYE emulsified in 0.5 ml of complete Freund adjuvant. Subsequently, 1.0 mg of MCYE or 3.0 mg of YCYE was injected biweekly. Localized infections were produced by biweekly subcutaneous injections of 10^8 viable yeast-phase organisms. Three groups of rabbits were immunized three times each week for 4 weeks with the following *C. albicans* antigens: (i) 10^8 viable clinical yeast isolate 737, injected subcutaneously; (ii) 10^8 viable clinical yeast isolate 738, injected subcutaneously; (iii) 10^8 Formalin-killed mycelial-phase type B strain 792 in 0.5 ml of complete Freund adjuvant, injected intramuscularly. Rabbits were bled 10 days after the last injections.

Indirect fluorescent-antibody (IFA) slide test. To each well of Teflon-coated, 12-well Microslides (Carlson Scientific), 10^6 organisms suspended in 10 μl of PBS were added, air dried, rinsed briefly in PBS, and dried with a jet of air. Typically, 10-μl aliquots of sera diluted 1:20 or 1:40 in PBS were applied to each well and incubated at 37°C for 30 min in a humidified box. Slides were rinsed in PBS with agitation for 15 min and air dried. Then 10 μl of fluorescein-tagged goat anti-rabbit immunoglobulin G (IgG) or anti-human IgG (Cappel Laboratories, Cochranville, Pa.) diluted 1:160 to 1:320 in PBS–0.05% Tween 20 (Sigma Chemical Co., St. Louis, Mo.) was applied to each well and incubated at 37°C for 30 min. The fluoresceinated antibodies were absorbed with *C. albicans* to eliminate anti-*Candida* antibodies. After a 15-min rinse, buffered glycerin mounting fluid (pH 9) was added to each well, and a cover slip was applied for examination under oil immersion at 400× magnification, using a Zeiss microscope equipped with a BG12 primary filter and a 50/-(II 10) secondary filter. Fluorescein-tagged antibody or test serum was omitted from some wells as controls. Normal rabbit serum was substituted for test serum to control for nonspecific staining. No significant qualitative or quantitative differences were noted between slides prepared with live or Formalin-killed organisms. Staining of the blastospore segment and the germ tube segment of the mycelial-phase organism was assessed individually and scored as follow: ++, brightly stained; +, weakly stained; +−, barely visible; 0, unstained.

To assess the stability of surface antigens, some slides were prepared by using Formalin-killed organisms previously heated to 63°C for 6 h. These slides, as well as conventional B311 slides, were incubated for 4 h at 23°C with either 0.75% chymotrypsin or trypsin in PBS and then were washed with PBS for 30 min. A standard IFA test was then performed.

A modified IFA slide test was employed to assess binding inhibition of specific antibodies by soluble extracts dissolved in PBS. DTT and KOH extracts from either phase were dissolved at 0.1, 1.0, and 5.0 mg/ml. Type A and type B CWM were prepared at 1.0 and 5.0 mg/ml, respectively. MCYE was dissolved at 1.0, 5.0, 10.0, and 20.0 mg/ml, and YCYE was dissolved at 1.0, 5.0, 10.0, and 40.0 mg/ml. Aliquots (10 μl) of extract solution were added to individual wells, followed by the addition of 10 μl of diluted serum and completion of a standard assay. For controls, bovine serum albumin (30 mg/ml) was substituted for inhibitor, or inhibitor was added but PBS was substituted for the test serum. Inhibition results were confirmed by a blinded observer.

Preparation of human tissue slices. Autopsy or biopsy specimens from cases of invasive candidiasis were fixed, mounted in paraffin, and sectioned with a microtome. Slides bearing the sections were soaked in xylene and then passed through a graded series of alcohol. The slides were kept moist until application of sera and completion of a standard IFA test. Slides were counterstained for 5 min with 0.1% Evans blue and rinsed with PBS.

Antibody absorptions. Anti-CWM activity was eliminated by adding 5.0 mg of type A CWM per ml, agitating at room

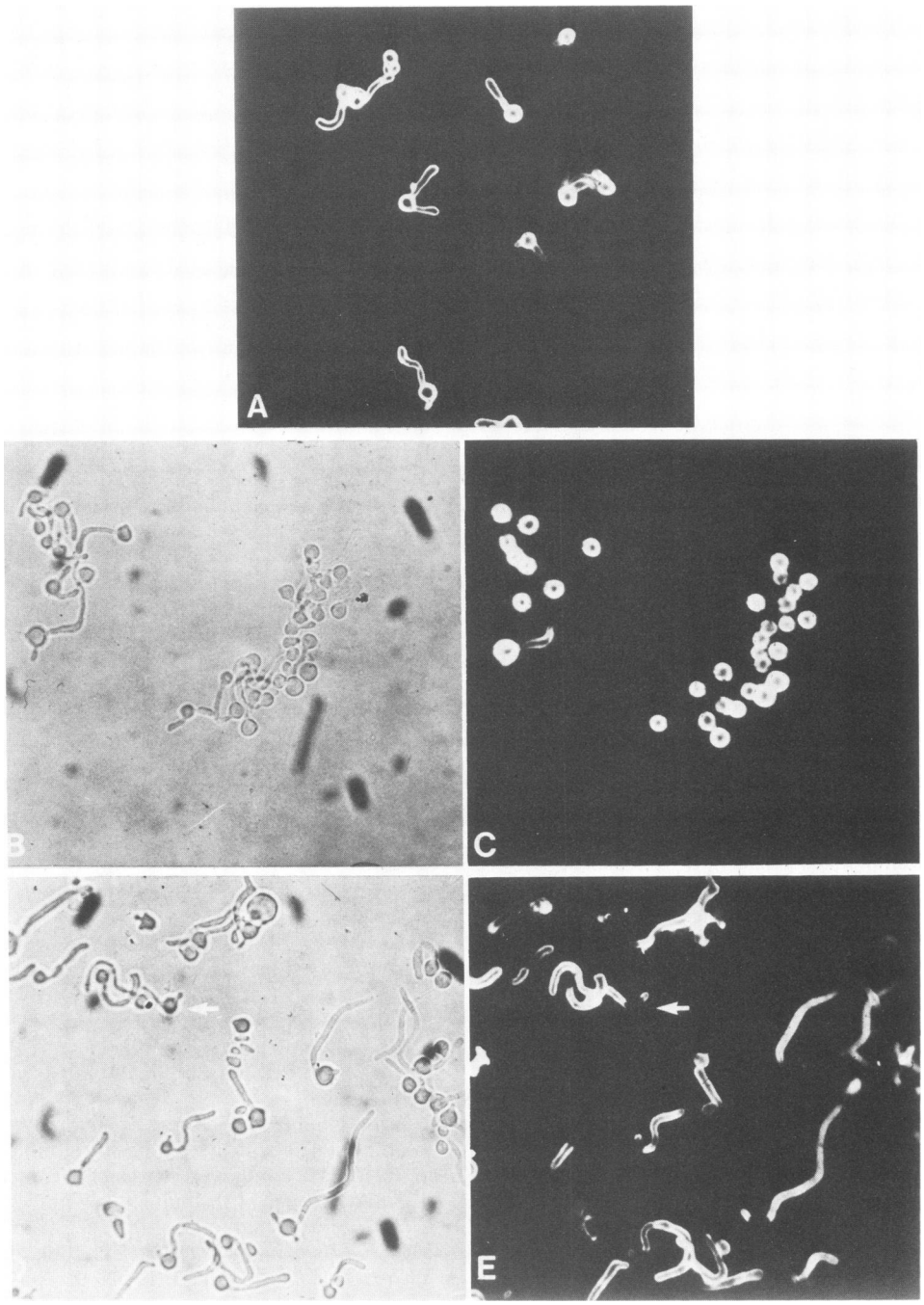

FIG. 1. Bright-field and fluorescence photographs from IFA studies of mycelial-phase *C. albicans* B311. Bright-field and fluorescence photos were exposed for 10 s and 10 min, respectively, under oil immersion at 400× magnification. Sera were diluted 1:40. (A) Fluorescence of organisms incubated with unabsorbed anti-FKYB311. (B and C) Bright-field and fluorescence illumination, respectively, of the same microscopic field, stained with serum raised against YCYE. (D and E) Bright-field and fluorescence illumination, respectively, of the same microscopic field, stained with FKYB311-absorbed anti-FKMB311. The arrow in (E) points to one of many blastospore segments not visible under fluorescence. Note retention of bright staining of germ tube segments in (E).

temperature for 2 to 4 h or overnight at 4°C, and centrifuging sera to remove precipitates. Removal of anti-mannan was documented by an indirect enzyme-linked immunosorbent assay (13). Sera were absorbed in an identical manner with 2.5 mg of mycelial-phase DTT extract per ml or 5.0 mg of yeast-phase DTT extract, YCYE, or MCYE per ml.

FKY or FKM organisms were used for absorption by adding 0.5×10^9 to 5.0×10^9 cells to 1-ml aliquots of sera and agitating at room temperature for 2 to 4 h or overnight at 4°C and centrifuging sera to remove the absorbant. Some sera were absorbed with 10^{11} yeast-phase organisms per ml. Some sera were absorbed with CWM as described above and then with whole organisms.

Immunoelectrophoresis. Crossed immunoelectrophoresis, crossed line immunoelectrophoresis, and tandem crossed immunoelectrophoresis were performed by modifications of

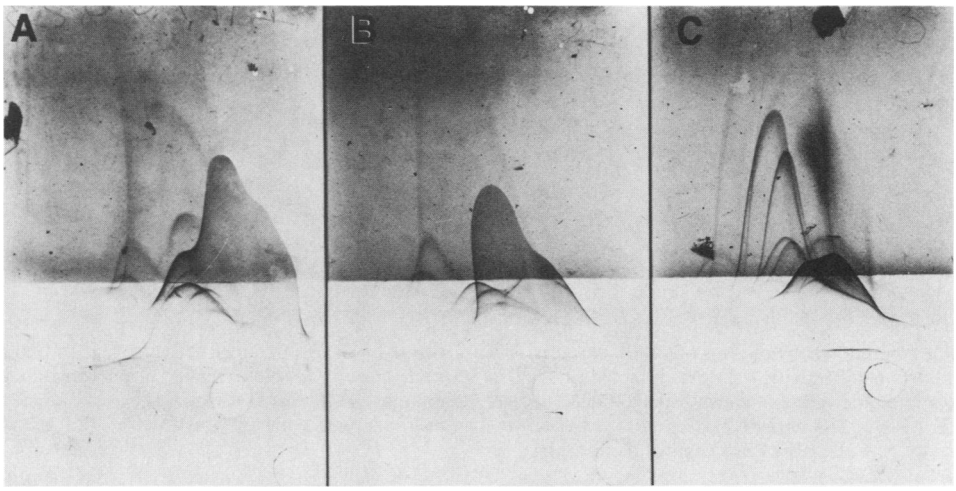

FIG. 2. Tandem crossed immunoelectrophoresis of extracts of *C. albicans* B311 against anti-FKMB311. (A) Yeast-phase DTT extract (400 μg) (upper well) and 400 μg of mycelial-phase DTT extract (lower well); (B) 100 μg of MCYE (upper well) and 400 μg of DTT extract of mycelial-phase organisms (lower well); (C) 100 μg of MCYE (upper well) and 400 μg of DTT extract of yeast phase (lower well).

the methods of Weeke (44), Guinet and Gabriel (15), and Kroll (24, 25), respectively, using glass plates (5.0 by 7.5 cm) and 1% agarose (type I; Sigma) in barbital buffer (pH 8.6). First-dimension electrophoresis was conducted at 10 V/cm for 45 min, and second-dimension electrophoresis was conducted at 2 V/cm for 18 h. The first-dimension anode was to the left of each plate, and the second-dimension anode was at the top of each plate. Each second-dimension gel contained 10.8 μl of antiserum per cm^2. Gels were stained with Coomassie brilliant blue R-250.

Protein determination. Protein concentrations were determined by the Folin phenol method (27), using bovine serum albumin as standard.

RESULTS

IFA staining of *C. albicans* B311. Staining of the blastospore segment by anti-Formalin-killed *C. albicans* B311 (anti-FKYB311) was more intense than staining of the germ tube segment, as noted by others (2, 41) (Fig. 1A). Staining was eliminated entirely when anti-FKYB311 was absorbed with yeast-phase B311 cells. After absorption with yeast-phase organisms, sera obtained from rabbits given localized infections with viable yeast-phase *C. albicans* organisms or from animals immunized with FKMB311 organisms (anti-FKMB311) both stained only the germ tube segment of the targets (Fig. 1D and E). Thus, during an infection, *C. albicans* expresses antigens that are similar to those found on germ tubes of mycelial-phase organisms grown in vitro. Retention of germ tube segment staining was seen with yeast-absorbed anti-MCYE, whereas anti-YCYE stained the germ tube segment very poorly, even when unabsorbed (Fig. 1B and C). Absorption of anti-FKMB311 with type A CWM resulted in a fourfold reduction in titer, but staining of both segments was retained. When sera were absorbed with MCYE, staining of the germ tube segment was reduced. Staining of the blastospore segment was reduced with sera absorbed with YCYE. We will use the term germ tube segment antigens to refer to antigens expressed primarily on the surface of mycelial-phase *C. albicans* organisms. For convenience, we will define blastospore segment antigens as those capable of binding antibodies responsible for IFA staining of blastospore segments, even though these antigens may also be expressed on the surface of germ tubes.

Absorption of high-titer anti-FKMB311 with 5×10^9 mycelial-phase organisms per ml resulted in complete loss of IFA staining. Bright staining of the germ tube was retained when identical serum was absorbed with 5×10^9 yeast-phase organisms per ml. However, absorption with 10^{11} yeast-phase organisms per ml resulted in complete loss of staining. Nonspecific absorption of immunoglobulin by huge numbers of organisms might explain this, but negligible reduction in staining was noted when identical serum was absorbed with 10^{11} *C. tropicalis* yeast cells. These data suggest that germ tube antigens are expressed on the surface of yeast-phase organisms, but in minute quantities compared with germ tubes.

Cell wall materials from *C. albicans* B311 were extracted by two methods, namely, treatment with the reducing agent DTT, which has been shown to release components from the outer portion of the cell wall (1), and a more severe treatment with KOH. When employed in IFA inhibition assays, mycelial-phase DTT extract solutions at 1.0 and 5.0 mg/ml significantly inhibited staining of germ tube segment by anti-FKMB311 serum. In contrast, a yeast-phase DTT extract, KOH extracts from either phase, and a bovine serum albumin solution failed to inhibit germ tube staining.

When cytoplasmic extracts were used as inhibitors, 5.0 mg of MCYE per ml effected almost complete elimination of staining of the germ tube segment by yeast-phase-absorbed anti-FKMB311. In contrast, 40.0 mg of YCYE per ml did not alter staining of the germ tube segment by this same absorbed serum. As protein contents of the MCYE and YCYE were 61 and 7.9%, respectively, YCYE was ineffective at blocking germ tube segment staining, even when equivalent concentrations of protein were compared. YCYE blocked staining of the blastospore segment by anti-FKMB311, but bovine serum albumin had no observable effect. Neither type A nor type B CWM inhibited staining of the germ tube segment by absorbed anti-FKMB311. Presence of surface antigens in the cytoplasmic extracts capable of inhibiting staining could reflect synthesis of these surface antigens within the cytoplasm. Alternatively, surface antigens may be shed into the cytoplasmic extracts during extraction.

Anti-FKMB311 was absorbed with yeast cells and CWM, ensuring that reaction of antibodies with the germ tube segment antigens alone would be assessed. Heating cells to

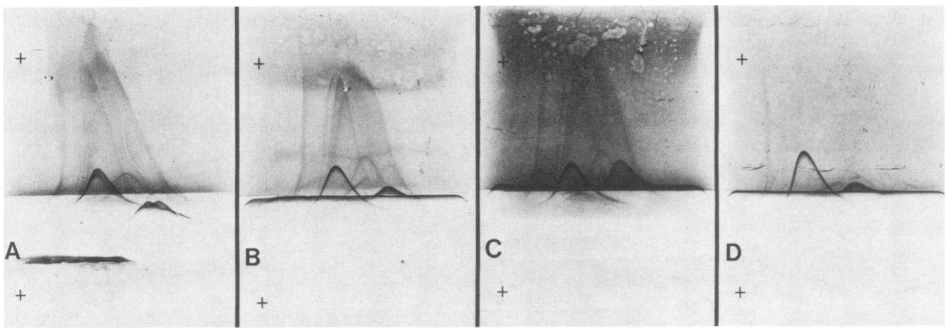

FIG. 3. Crossed line immunoelectrophoresis of DTT extracts of *C. albicans* B311 against absorbed and unabsorbed anti-FKMB311. The circular well of each plate was filled with 400 μg of mycelial-phase DTT extract. The control plate (A) had no antigen added to gel incorporated into the line well, but in the other plates this well was loaded with a gel containing 600 μg of yeast-phase DTT extract. Second-dimension gels contained (A) anti-FKMB311, (B) anti-FKMB311, (C) anti-FKMB311 absorbed with 5 mg of yeast-phase DTT extract per ml, (D) anti-FKMB311 absorbed with 5×10^9 FKYB311 organisms per ml.

63°C for 6 h produced a very minor reduction in staining intensity. Incubation of untreated and heat-treated cells with 0.75% trypsin or chymotrypsin for 4 h had no effect beyond that seen with heat alone.

IFA staining of other *C. albicans* strains and *Candida* species. Slides bearing the mycelial phase of several clinical and laboratory strains of *C. albicans* were incubated with B311-absorbed anti-FKMB311. Five of the six heterologous strains tested stained exactly as did B311, indicating that antigens similar or identical to the germ tube segment antigens of B311 are expressed on the germ tube segments of other strains.

Antisera from four pairs of rabbits, each pair of which had been immunized with a different strain of *C. albicans*, were absorbed with yeast-phase organisms from autologous and heterologous strains. When absorbed with yeast-phase organisms of the immunizing strain, blastospore staining of the immunizing strain was eliminated or markedly reduced, whereas staining of germ tube segments was retained. Thus, for each of these four strains, one or more antigens were expressed primarily on the germ tube and stimulated an antibody response. In addition, these absorption studies indicated that the germ tube antigens demonstrated for B311 were either absent or present in insignificant amounts on the yeast phases of the other strains tested, because in no case did absorption of anti-FKMB311 with these heterologous strains eliminate staining of the germ tube segment.

The expression of antigens on other yeasts was studied. Aliquots (1 ml) of anti-FKMB311 were absorbed with 10^9 Formalin-killed yeast forms of *S. cerevisiae*, *C. tropicalis*, *C. guillermondii*, *C. stellatoidea*, or *C. albicans* B311. Only *C. albicans*- and *C. stellatoidea*-absorbed sera produced staining limited to the germ tube segment, indicating that of the non-*C. albicans* species tested, only *C. stellatoidea* had sufficient expression of blastospore segment antigens to absorb antibodies to these antigens. In another experiment, 1-ml aliquots of anti-FKMB311 were absorbed with 10^9 yeast cells of *S. cerevisiae*, *C. tropicalis*, *C. guillermondii*, or *C. stellatoidea* followed by absorption with 10^9 FKYB311 organisms. Staining of germ tube segments by these sera was not significantly different from staining by sera absorbed with FKYB311 alone, indicating that these species did not express significant amounts of germ tube antigens on the surface of their yeast-phase cells. When anti-FKMB311 was absorbed with yeast cells of *C. stellatoidea* and incubated with mycelial-phase *C. stellatoidea*, staining limited to the germ tube segment was seen, indicating that the germ tube segment of *C. stellatoidea* expressed antigens cross-reactive with germ tube antigens of *C. albicans*.

Immunoelectrophoresis. KOH and DTT extracts of yeast- and mycelial-phase *C. albicans* B311 were analyzed by crossed immunoelectrophoresis. Although KOH treatment solubilized larger amounts of cell wall material from both phases than did DTT, no immunoreactive material was demonstrated in immunoelectrophoresis by either anti-FKMB311 or anti-FKYB311. In contrast, anti-FKMB311 recognized 12 antigens in mycelial-phase DTT extracts and up to 7 antigens in yeast-phase DTT extracts. Despite intense exposure to yeast-phase organisms, anti-yeast-phase sera produced only two faint precipitin arcs when reacted with DTT extracts from either phase of *C. albicans*. Mycelial-phase DTT extract, yeast-phase DTT extract, and MCYE were compared by reaction against anti-FKMB311 in tandem crossed immunoelectrophoresis (Fig. 2). A dense, cross-reactive arc from all three extracts was seen. In view of this, yeast-phase DTT extract and mycelial-phase DTT extract were analyzed by crossed line immunoelectrophoresis against anti-FKMB311 (Fig. 3). The cross-reactive component in mycelial-phase DTT extract was evident on all plates. The dense peak produced by this component in mycelial-phase DTT extract fused with the dense line produced by yeast-phase DTT extract; however, a line produced by the yeast phase was clearly seen beneath this peak (Fig. 3B). This might indicate that some molecules of the component in the mycelial-phase extract lacked antigenic determinants expressed on the component in the yeast-phase extract. Alternatively, more than one component in the yeast-phase extract may have the same electrophoretic mobility under the conditions used. Some increase in the height of the cross-reactive peak relative to other peaks was noted, which is consistent with absorption from anti-FKMB311 of some of the antibody reactive with antigenic determinants of this component (Fig. 3C). Absorption of anti-FKMB311 with FKY eliminated many peaks from the mycelial-phase DTT extract (Fig. 3D). Nevertheless, one prominent and several faint mycelial-phase DTT arcs remained which crossed the precipitin line produced by yeast-phase DTT extract. Antibodies reactive with the prominent cross-reacting determinant remained in the absorbed antiserum. Exclusive germ tube segment staining by IFA was seen with these absorbed sera. Together, these data indicate that mycelial-phase and yeast-phase DTT extracts contained

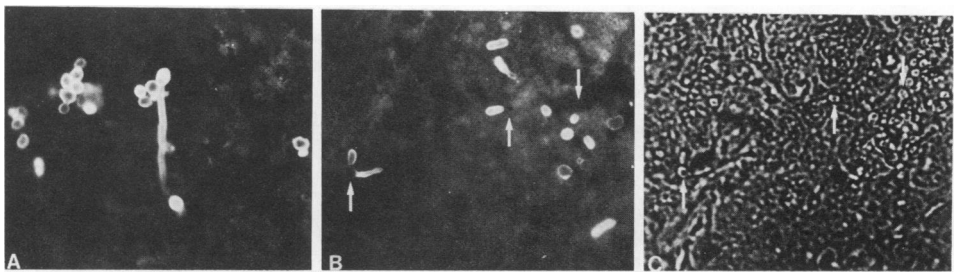

FIG. 4. Bright-field and fluorescence photographs of IFA staining of human tissue slices. (A) Staining of blastospore and germ tube segments is evident in tissue stained with anti-FKMB311. (B and C) Fluorescence and bright-field photographs, respectively, of the same microscopic field. Arrows point to blastospore segments visible under white light in (C) but not under UV light in (B). Tissue slices were incubated with undiluted yeast-absorbed anti-FKMB311. Photography was performed under the same conditions as those for Fig. 1.

cross-reactive as well as non-cross-reactive antigens. The antigenic determinants were exposed on the germ tube surface and stimulated an antibody response. However, these determinants were not significantly expressed on the yeast-phase surface.

Studies of sera and infected tissues from humans. Experiments were performed to determine whether germ tube segment antigens were expressed on *Candida* spp. invading human tissues and whether these antigens could stimulate specific antibody responses in humans. Sections of tissues infected with *Candida* spp. were obtained from patients by biopsy or necropsy and were stained by the IFA technique with FKYB311-absorbed anti-FKMB311. Figure 4 shows staining patterns produced by IFA staining of tissues with absorbed and unabsorbed anti-FKMB311. Staining limited to the germ tube segment was seen in 14 of 17 specimens incubated with absorbed sera.

Seventeen sera from 14 patients with proven disseminated candidiasis were compared by IFA testing with 30 sera randomly chosen from our clinical laboratory. All sera were diluted 1:20 and incubated in the standard manner, using FKMB311 organisms as targets. Unabsorbed sera from all patients with candidiasis brightly stained both segments of the mycelial-phase organism. Only 3 of 30 unabsorbed random control sera produced this pattern of staining; the remainder of these sera preferentially stained the blastospore segment. When appropriately absorbed, 14 of 17 sera from patients with candidiasis stained germ tubes primarily or exclusively.

DISCUSSION

Early studies employing agglutination assays divided 71 yeast-phase *C. albicans* strains into types A and B (17). Type A cells contained all surface antigens demonstrable on type B strains plus an additional antigen or antigens. When yeast-phase cell wall extracts were reacted in double immunodiffusion plates with anti-whole killed yeast-phase *C. albicans*, the major antigen identified was mannan (39), which was capable of inhibiting agglutination of yeast-phase cells (18). Despite its abundance on the *Candida* surface, mannan does not appear related to the germ tube antigens identified by our experiments. Brightly visible germ tube staining was retained during IFA tests with mannan-absorbed sera as well as during IFA inhibition studies in which high concentrations of mannan were used as a putative inhibitor. In comparing surface staining of both phases of *C. albicans*, Venezia and Lachapelle (43) found no differences when organisms were incubated with ferritin-conjugated, anti-killed yeast-phase *C. albicans*. They concluded that surface antigens on the two forms were homogeneous. In view of our data from this study showing that yeast-phase organisms did not stimulate an antibody response to germ tube antigens, preferential staining of the germ tube segment with their sera would not be expected. Electron microscopic studies involving the mannan-specific lectin concanavalin A have demonstrated a continuous distribution of mannan on the outer surfaces of mycelial-phase organisms (42). This is most likely the antigen to which their sera were directed.

By indirect immunofluorescence, using diluted sera from patients with candidiasis, more intense staining of *Candida* germ tubes than of blastospores has been reported (16, 20). Using yeast-absorbed sera from rabbits immunized with viable type B yeast, Poulain et al. (34) reported specific germ tube staining of in vitro and in vivo germ tube forms. Our results were consistent with these observations. When these authors absorbed anti-killed type A yeast-phase sera with type B yeast, the resultant absorbed sera continued to stain type B germ tubes. The authors concluded that under some conditions, type B cells might express antigens thought to be type A specific. We did not observe preferential staining of the germ tube with sera from rabbits immunized with killed yeast. Whereas Poulain et al. used a type B yeast to absorb anti-type A yeast sera, we absorbed with the homologous strain. Strain or type variations or both may explain the disparate results. Alternatively, undetected contamination of their immunogen with mycelial-phase organisms could explain their results.

Manning and Mitchell (28) found cytoplasmic antigens unique to the mycelial phase of one strain of *C. albicans* but demonstrated the same antigens in the yeast-phase extract of another strain. We demonstrated germ tube antigens on the germ tubes of a variety of strains but did not find these antigens in significant amounts on yeast forms from any other strain or species tested. Preferential germ tube staining of *C. stellatoidea* was seen, but *C. stellatoidea* may be a variant of *C. albicans* (3). The results from IFA studies of human tissue slices provide additional evidence that these antigens are present on the germ tubes of many strains in nature.

Whereas absorption of high-titer anti-FKMB311 with 5×10^9 organisms of FKMB311 per ml was sufficient to eliminate visible immunofluorescence of germ tubes, absorption with 10^{11} organisms of FKYB311 per ml was necessary for elimination of immunofluorescence. This implies the presence on the yeast surface of tiny amounts of germ tube segment antigens. Demonstration by immunoelectrophoresis of cross-reacting antigens from DTT extracts supports this conclusion. Ho et al. (19) demonstrated an antigen (component II) obtained from sonic extracts of whole yeast- and

mycelial-phase organisms that reacted against anti-mycelial-phase sera but not against anti-yeast-phase sera. They speculated that this antigen was exposed on germ tubes but was present in a cryptic state on yeast-phase organisms. Different methodologies limit comparisons, but the cross-reactive antigens demonstrated by immunoelectrophoresis (Fig. 2 and 3) may be closely related to component II.

Crossed line immunoelectrophoresis of DTT extracts revealed, in addition to the cross-reacting antigens, one prominent and several faintly staining non-cross-reactive arcs when incubated with yeast-absorbed anti-FKMB311 (Fig. 3D). This implies antigens expressed uniquely on the surface of the germ tube. However, were unique antigens exposed in significant quantities on germ tubes, even exhaustive absorption with yeast-phase organisms would not eliminate germ tube staining. As noted above, absorption with 10^{11} *C. albicans* organisms effected complete elimination of staining. Significant differences in sensitivity of assay systems might explain these conflicting data. An alternative explanation may be that the antigens appearing to be unique to the germ tube by crossed line immunoelectrophoresis actually have cross-reactive counterparts present in tiny amounts on the yeast-phase surface which either are not extracted by DTT or lose their cross-reactivity during extraction.

During saprophytic colonization, *C. albicans* exists almost exclusively as the yeast phase, but with tissue invasion, a combination of yeast forms, pseudohyphae, and mycelial-phase organisms is almost universally found (32, 46). Several authors have found that mycelial-phase organisms adhere to epithelial surfaces better than do yeast-phase organisms (23, 37, 38). Polymorphonuclear leukocytes have been demonstrated to attach to, damage, and probably kill hyphae and pseudohyphae (7). From dying hyphal and pseudohyphal forms, a low-molecular-weight polypeptide was identified that inhibited attachment of leukocytes to *C. albicans* (8). These various studies indicate that alterations of the surface of *C. albicans* by germination are important in pathogenesis. What role, if any, the germ tube segment antigens play in these processes remains to be elucidated. Purification and characterization of these antigens would be essential to such investigations. Purified germ tube segment antigens could also be employed in developing new serodiagnostic assays that might be more specific for invasive disease than currently available tests.

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