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Partial Characterization of a *Candida albicans* Fimbrial Adhesin

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***Candida albicans* is the primary etiologic agent of candidiasis, a disease that can vary from superficial mucosal lesions to life-threatening systemic or disseminated diseases. Strains of *C. albicans* have been reported to possess long, thin filamentous protein cell surface appendages termed fimbriae (R. B. Gardiner, M. Canton, and A. W. Day, Bot. Gaz. 143:534–541, 1982). These fimbriae were isolated, purified, and partially characterized. The major structural subunit of the fimbriae is a glycoprotein which consists of 80 to 85% carbohydrate (consisting primarily of D-mannose) and 10 to 15% protein. The molecular weight of the glycosylated fimbrial subunit is approximately 66,000, while unglycosylated protein has an approximate molecular weight of 8,644. The fimbriae function as adhesins mediating *C. albicans* binding to human buccal epithelial cells. Amino acid analysis of the purified fimbrial subunit indicates that the fimbrial subunit is composed of 50% hydrophobic amino acid residues. The N terminus of the fimbrial subunit is blocked to N-terminal sequencing.**

Candida albicans, a dimorphic, imperfect yeast, is a significant human pathogen that can cause superficial or invasive diseases (49). *C. albicans* can be isolated from the oral cavity of approximately 40% of healthy, asymptomatic individuals. *C. albicans* is considered to be an opportunistic pathogen that primarily infects immunocompromised or immunosuppressed individuals (6, 45, 47). The initial stage of candidiasis is thought to be the adherence of the yeast to a mucosal surface (4, 12, 28, 42, 43), and a strain's ability to adhere to human buccal epithelial cells (BECs) has been correlated with virulence in animal infection models (22, 34). *C. albicans* appears to utilize multiple adherence mechanisms, as several adhesins have been described (8, 29, 49). Critchley and Douglas (7, 11) and Tosh and Douglas (50) have described a *C. albicans* lectin-like adhesin that binds to host-associated carbohydrates which contain fucose or *N*-acetylglucosamine. Fukazawa et al. (14) and Miyakawa and coworkers (38) have reported that a *C. albicans* cell surface carbohydrate can interact specifically with a host-associated lectin and thus function as an adhesin. *C. albicans* cell surface hydrophobicity may also contribute significantly to the adherence to both mucosal epithelial cells and inert surfaces (17–22). None of the putative *C. albicans* adhesins have been extensively characterized to date.

C. albicans fimbriae were initially described by Gardiner et al. (15) but have never been extensively characterized. We have thus isolated and partially characterized *C. albicans* fimbriae both morphologically and biochemically. The fimbriae function as a significant adhesin which mediates *C. albicans* binding to human BECs.

MATERIALS AND METHODS

Strain and culture conditions. *C. albicans* 40 was obtained from the trachea of an intubated intensive care unit patient at Toronto General Hospital. The isolate has been maintained at −70°C in 40% glycerol containing 3% trisodium citrate following the initial isolation and microbiological characterization of the isolate. The isolate was subsequently recovered on Sabouraud dextrose (SAB) agar (GIBCO) at 37°C for 18 h. *C. albicans* was then recultured on SAB agar plates for 18 h at 37°C, harvested in 3 ml of 10 mM phosphate-buffered saline (PBS) (pH 7.2), and utilized to inoculate trays (30 by 22 cm) of SAB agar which were then incubated for 5 days at 37°C before cells were harvested.

Purification of *C. albicans* fimbriae. Fimbriae were purified from the yeast phase of *C. albicans. C. albicans* cells were harvested from the agar surface by gentle scraping with a bent glass rod. Harvested cells were suspended in a minimal volume (50 ml per tray) of preparation buffer (10 mM sodium phosphate saline [pH 7.2] containing 1 mM $CaCl_2$ and 1 mM phenylmethylsulfonyl fluoride). Harvested cells were washed three times with 500 ml of preparation buffer by centrifugation (12,000 × *g* for 20 min at 4°C). Fimbriae were sheared from the cell surface by gentle homogenization (four 45-s cycles) with a Brinkmann homogenizer. The cells were removed by centrifugation (12,000 × *g* for 20 min) and by subsequent filtration of the supernatant through a 0.45-μm-pore-size filter (Millex-PF; Millipore). The supernatant was concentrated approximately 10-fold with polyethylene glycol (molecular weight, 8,000). The concentrated fimbrial preparation was dialyzed overnight at 4°C against preparation buffer. This material was termed crude fimbriae (CF). The CF preparation was fractionated by size exclusion chromatography (SEC)–high-performance liquid chromatography (HPLC) using an isocratic gradient (flow rate = 0.5 ml/min; column, Waters Protein-PAK 300 SW, 10 μm) with preparation buffer as the solvent. The material that was eluted in the void volume was collected, concentrated with polyethylene glycol, and dialyzed against preparation buffer. This material was termed semienriched fimbriae and was rechromatographed under identical conditions. The peak which corresponded with the void volume of the column was again collected, concentrated, and dialyzed against preparation buffer. This fraction was termed enriched fimbriae (EF).

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Determination of *C. albicans* fimbrial preparation protein concentrations. Protein concentrations of CF and EF were determined by a bicinchoninic acid protein assay (Pierce) described by Smith et al. (48) with bovine serum albumin (BSA) employed as the protein standard to generate a standard curve.

Amino acid composition of the *C. albicans* fimbrial subunit. Amino acid analysis was performed on *C. albicans* fimbriae electroeluted from a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (described below) that was subsequently purified by reversed-phase chromatography. CF were separated by SDS-PAGE. The band on the gel containing the protein of interest was cut according to the size of the protein (the protein of interest had been previously identified by Western blotting [immunoblotting] analysis as described below). The gel slices containing the fimbrial subunits were washed (two times for 5 min each) with elution buffer (20 mM ammonium bicarbonate) by gently shaking at 50 rpm on a Gyrotory shaker model G2 (New Brunswick Scientific Co., New Brunswick, N.J.) for 30 min at room temperature. The gel slices were put into dialysis tubing (molecular weight cutoff of 6,000 to 8,000) and suspended in water. The proteins were electroeluted from the gel slices in 20 mM ammonium bicarbonate with an electroelution apparatus (Schleicher & Scheull Elutrap) by applying a constant voltage of 200 V for 5 h or 80 V overnight. The eluate was collected and dialyzed against deionized water.

The fimbrial subunits were further purified by reversed-phase HPLC (Aquapore C_4 column; 100 by 4.6 mm; 7-μm internal diameter) using a linear AB gradient (where solvent A is 0.05% aqueous trifluoroacetic acid and solvent B is 0.05% trifluoroacetic acid in acetonitrile) of 2% B per min at a flow rate of 1 ml/min. The eluate was collected and lyophilized. A small amount of the lyophilized fimbriae was hydrolyzed in a glass tube with 200 μl of 6 N HCl containing 0.1% (wt/vol) phenol at 110°C for 24 h in vacuo. The acid from the hydrolysate was removed by evaporation and resuspended in citrate buffer, pH 2.2, and the amino acid content was analyzed with a Beckman model 6300 amino acid analyzer. No attempt was made to analyze for total half-Cys or Trp, nor were the values for Ser and Thr corrected to take into account losses during hydrolysis.

Carbohydrate composition of *C. albicans* fimbriae. A phenol-sulfuric acid carbohydrate assay described by Dubois et al. (13) was used to determine the amount of carbohydrate present in the EF preparation. EF were diluted 1:10 with 2 N H_2SO_4. Diluted EF (0.5 ml) were added to 0.5 ml of a 5% solution of aqueous phenol and 2.5 ml of H_2SO_4 reagent (2.5 g of hydrazine sulfate in 1 liter of concentrated sulfuric acid) and mixed vigorously before incubation in the dark for 1 h at room temperature. The A_{490} of the reaction mixture was recorded. D-Mannose (Sigma) was dissolved in 2 N H_2SO_4 and employed as a standard (0 to 100 μg/ml).

Based on the known amount of EF used for the carbohydrate analysis, both the protein and carbohydrate contents in the *C. albicans* fimbriae could be used to determine the ratio of carbohydrate and protein.

The carbohydrate composition of the EF was investigated as described by Bryn and Jantzen (2, 3). Briefly, lyophilized carbohydrate samples were methanolyzed with dry 2 M HCl–methanol for 16 h at 85°C. The derivatization mixture (2 μl) was used directly. Samples were analyzed with a Varian Vista 6000 chromatograph equipped with a Varian CDS 401 data station and a flame ionization detector and with a J & W DB-5 (95% methylpolysiloxane, 5% phenylpolysiloxane) column (30 cm by 0.25 mm [internal diameter]) with helium carrier at a flow rate of 1 ml/min. The column was held isothermally for the initial 4 min at 90°C, then the temperature rose at 8°C/min to a maximum of 270°C. Authentic carbohydrate samples (Sigma) were derivatized and utilized as standards.

SDS-PAGE and Western blot analysis of *C. albicans* fimbrial preparations. SDS-PAGE was performed with 12.5% acrylamide gels in a minigel apparatus (Mini-Protein II Dual Slab Cell; Bio-Rad) as described by Laemmli (31). Samples were electrophoresed for 50 min at a constant voltage of 200 V with a model 1420A power supply (Bio-Rad). Gels were stained with Coomassie blue (R-250; Bio-Rad) or with silver stain (53).

Proteins on the SDS-PAGE gel were transferred to nitrocellulose membrane by the protocol of Towbin et al. (51) with a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The transfer was completed after 30 min under a constant current of 300 mA (model 200/2.0 power supply; Bio-Rad). Excess binding sites on the membrane were blocked by incubation of the blots overnight at 4°C with a blocking solution consisting of 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 0.05% (vol/vol) Nonidet P-40, 0.25% (wt/vol) gelatin, and 3% (wt/vol) BSA. The membrane was washed twice at room temperature with 10 mM Tris-HCl buffer (pH 7.5) containing 0.1% Tween 20 and 0.05% (wt/vol) BSA (TTBS). The membrane was cut into 0.5-cm strips and placed into slots in transfer plates. Mouse anti-EF ascitic fluid, Fm16, and normal mouse immunoglobulin G (IgG; 10.3 mg/ml; Jackson ImmunoResearch Laboratory) were diluted with TTBS (1:500), added to the respective strips, and incubated for an hour at 37°C in an incubator shaker (model G25 Gyrotory shaker; New Brunswick Scientific) set at 100 rpm. The strips were washed three times with TTBS. A goat anti-mouse heavy- and light-chain IgG–alkaline phosphatase conjugate (Jackson Laboratories) diluted 1:10,000 with TTBS was incubated as described above. The strips were washed three times with TTBS and then given a final wash with Tris-buffered saline. Antibody binding was visualized by the addition of alkaline phosphatase substrates (nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate dissolved in 100 mM Tris-HCl, pH 9.5, containing 100 mM NaCl and 5 mM $MgCl_2$). Color development was stopped by rinsing the nitrocellulose strips with deionized water.

Production of the antifimbria monoclonal antibody, Fm16. Anti-*C. albicans* fimbria monoclonal antibody Fm16, was produced by a hybridoma technique previously employed to obtain *Pseudomonas aeruginosa* pilus monoclonal antibodies (10). BALB/c female mice (Charles River Breeding Laboratories Inc.) were immunized on days 1, 8, 15, 32, and 46 with 10 μg of EF in 1% (wt/vol) $Al(OH)_3$. The EF were first denatured by boiling in a 1% (wt/vol) SDS and 1 mM β-mercaptoethanol. Animals were exsanguinated, and the antibody titers were determined by enzyme-linked immunosorbent assays (ELISA) with semienriched fimbriae (10 μg/ml) as the coating antigens in the microtiter wells (100 μl per well).

Following the development of high-titer antibodies, three mice were sacrificed and their spleens were removed aseptically. The mouse myeloma used for the production of hybridoma clones was NS1. The NS1 cell line was cultured in high-glucose Dulbecco modified Eagle medium supplemented with 2 mM L-glutamine and 10% (vol/vol) fetal calf serum (GIBCO) at 37°C in the presence of 5% CO_2. Cells were passed every 48 h at a split ratio of 1:5 or 1:4. Production and selection of hybridomas were carried out as described by Irvin and Ceri (23), except that the medium was not supplemented with β-mercaptoethanol. Clones were selected for their ability to synthesize anti-*C. albicans* fimbria antibodies as determined by ELISA employing EF as the antigens. Positive clones had

A_{405} values that were double or greater than control levels. These results were confirmed by Western blots. Positive hybridoma clones were scaled up into 5 ml of Dulbecco modified Eagle medium supplemented with 20% (vol/vol) fetal calf serum, 2 mM L-glutamine, hypoxanthine, aminopterin, and thymidine. Clones were frozen and subsequently subcloned twice in semisolid agarose (25, 26). One particular monoclonal antibody, Fm16, was chosen for further analysis. Fm16 is an IgG2a(κ), according to isotyping results obtained with SBA Clonotyping System II (Southern Biotechnology Associates, Inc., Birmingham, Ala.). Ascites tumors were produced by injecting 10^6 hybridoma cells into pristane-primed BALB/c male mice (30). Ascites fluid was recovered daily with a 25-gauge needle following the development of an ascites tumor. Typically, 15 ml of ascites fluid was collected over a period of 7 to 10 days.

ELISA. Polystyrene microtiter wells (Nunc) were coated with semienriched fimbriae (10 μg/ml in 0.01 M carbonate buffer, pH 9.5) by an overnight incubation at 4°C. The wells were washed three times (250 μl per well) with PBS (pH 7.4) supplemented with 0.05% (wt/vol) BSA (buffer A). Excess binding sites were blocked by incubation at 37°C for 1 h with 5% (wt/vol) BSA in PBS, pH 7.4. Wells were washed three times with 250 μl of buffer A per well. Aliquots (100 μl per well) of serially diluted mouse anti-EF monoclonal antibodies and antifimbria polyclonal sera (obtained from immunized mice that were sacrificed for fusion of mouse spleen and the NS1 cell line) were added to each well and incubated at 37°C for 2 h. Rabbit polyclonal anti-*Ustilago violacea* fimbria antiserum was a gift from A. Castle (Brock University, St. Catherines, Ontario, Canada). Wells were washed five times with 250 μl of buffer A per well. Antibody binding to EF was assessed by adding 100 μl of (1:5,000) goat anti-mouse or goat anti-rabbit heavy- and light-chain IgG–peroxidase conjugates (Jackson Laboratories) per well. Following a 1-h incubation at 37°C, the wells were washed five times with 250 μl of buffer A per well, and a substrate solution containing 1 mM 2,2′-azido-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS) in 10 mM sodium citrate buffer, pH 4.2, containing 0.03% (vol/vol) hydrogen peroxide was added (125 μl per well). The reaction was stopped by the addition of 4 mM sodium azide (125 μl per well), and the A_{405} was recorded.

Agglutination assays. *C. albicans* yeast cells were cultured in M9 medium as described below except that no radiolabels were added into the medium. The cells were fixed with 1% (vol/vol) formalin in PBS (pH 7.4) by incubation for 1 h at room temperature. The cells were harvested by centrifugation and washed three times with PBS. The cell number was determined in a hemacytometer and adjusted to 2×10^7 cells per ml. Aliquots (50 μl) were dropped onto microscope glass slides. Aliquots (50 μl) of serially diluted antibodies (Fm16, anti-EF polyclonal antiserum, and normal mouse IgG) were added to the *C. albicans* on the slides and incubated for 10 min at 37°C. The agglutination of the yeast was scored by phase-contrast microscopy.

Electron microscopy of fimbriae. Fimbriae were diluted 1:100 with 10 mM sodium phosphate buffer, pH 7.2. A 20-μl drop of diluted fimbria solution was placed on a freshly prepared carbon-Formvar-coated 3-mm 200-mesh copper electron microscope grid (Fisher Scientific). The grid was blotted with Whatman no. 1 filter paper and then negatively stained with 1% (wt/vol) phosphotungstic acid at pH 7.0 for 10 s. The stain was removed by blotting, and the sample was examined with a Philips model 410 transmission electron microscope operating at an accelerating potential of 80 kV. Micrographs were recorded on Kodak electron microscope film 4489.

Scanning electron micrographs of fimbriae of yeast-phase *C. albicans* bound to human BECs were obtained according to the methods of Murakami et al. (39). Specimens (3 ml) were fixed with 2.5% (vol/vol) glutaraldehyde (J.B. EM Services Inc., Point Claire, Dorval, Quebec, Canada) in 0.1 M phosphate buffer, pH 7.3, and incubated overnight at 4°C. Samples were aliquoted in 1.5-ml Eppendorf tubes, centrifuged at 100 × *g* for 10 min, and washed three times for 20 min each time with 1.0 ml of phosphate buffer, pH 7.3. Samples were postfixed in 2% (wt/vol) osmium tetroxide in 0.1 M phosphate buffer, pH 7.3, for 1 h. The cells were washed by centrifugation as described above. Specimens were then resuspended in 1.0% (wt/vol) tannic acid in distilled water and incubated for 30 min at room temperature. The solution was removed by aspiration, and the cells were washed with water, then resuspended in 2% (wt/vol) aqueous osmium tetroxide for 1 h, and then washed with water. Specimens were then dehydrated in a graded series of ethanol to 100%. Samples were critical point dried and subsequently salted onto a standard Cambridge scanning electron microscope stub precoated with double-sided adhesive tape. Specimens were then directly examined in a Hitachi S 4000 field emission scanning electron microscope at an accelerating potential of 2.5 kV.

Adherence of *C. albicans* fimbriae to human BECs. Human BECs were collected from 10 healthy, nonsmoking male volunteers by gentle scraping of the buccal mucosal surface with wooden applicator sticks. These sticks were then agitated in 40 ml of PBS, pH 7.2, to remove the BECs. BECs were washed three times for 10 min each time with 10 ml of PBS by centrifugation at 2,000 × *g*. Cell clumps were removed by filtration through a 70-μm-pore-size nylon mesh (Spectrum; Cole-Parmer). The cell concentration was determined directly with a hemocytometer, and BECs were resuspended in PBS to a concentration of 2.0×10^5/ml.

The fimbrial adherence assay was performed using a Manifold filtration apparatus equipped with individual vacuum stopcocks (model FH 225V; Hoefer Scientific Instruments). Polycarbonate filters, 12-μm pore size (Nucleopore Costar Corp.), were preincubated overnight at 4°C with 50 ml of PBS, pH 7.2, containing 0.45% (vol/vol) Tween 20. The pretreated filters were placed into each chamber and washed with 2.5 ml of PBS. One milliliter containing 2.0×10^5 BECs in PBS was added to each chamber. EF (100 μl per chamber, ranging from 0 to 80 μg of fimbrial protein per ml) in PBS containing 0.05% (vol/vol) Tween 20 were added to each chamber and incubated with the BECs for 1.5 h at room temperature. Unbound fimbriae were removed with washes of 2.5 ml of PBS. Mouse anti-*C. albicans* fimbria monoclonal antibody (ascites fluid diluted 1:3,000 with PBS) was added to the BECs (1.2 ml per chamber) and incubated for 1.5 h at room temperature. BECs were washed five times with 2.5 ml of PBS. Goat anti-mouse heavy- and light-chain IgG–peroxidase conjugates (Jackson Laboratories) diluted 1:5,000 with PBS were added (1.0 ml per chamber) and incubated for another hour at room temperature. The cells were then washed seven times with 2.5 ml of PBS per chamber. The polycarbonate filters containing BECs were removed from the filtration manifold and placed into glass scintillation vials. The horseradish peroxidase substrate solution (ABTS) was added to each vial (1 ml per vial) and incubated for 30 min at room temperature on a shaker at 100 rpm. The reaction was stopped by the addition of 4 mM sodium azide (200 μl per vial). The substrate solution was pipetted into Eppendorf tubes and centrifuged at 5,000 × *g* for 3 min. Aliquots of the supernatants were pipetted into microtiter wells (200 μl per well), and the resulting A_{405} was recorded with a Titertek Multiskan Plus microplate recorder.

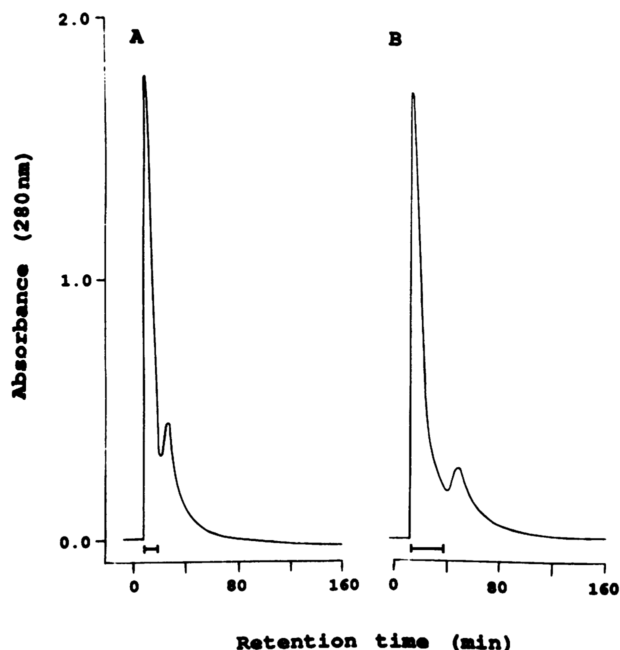

FIG. 1. Purification of fimbriae from an isolate of *C. albicans* associated with an extensive pulmonary infection of an intensive care unit patient. Fimbriae were purified by HPLC utilizing successive SEC with a Waters Protein-PAK 300 SW column (7.8 mm by 30 cm) equilibrated and eluted isocratically at 0.5 ml/min with 10 mM PBS, pH 7.2, containing 1 mM calcium chloride and 1 mM phenylmethylsulfonyl fluoride. (A) SEC-HPLC profile of material sheared by gentle homogenization from washed *C. albicans* cells cultured on SAB agar at 37°C for 5 days. The material which was eluted at the void volume (denoted by a solid bar) was collected (termed semienriched fimbriae) and rechromatographed under the same conditions. (B) SEC-HPLC profile of rechromatographed fimbriae. Fimbriae which were eluted at the void volume (solid bar) were termed EF (~95% purity).

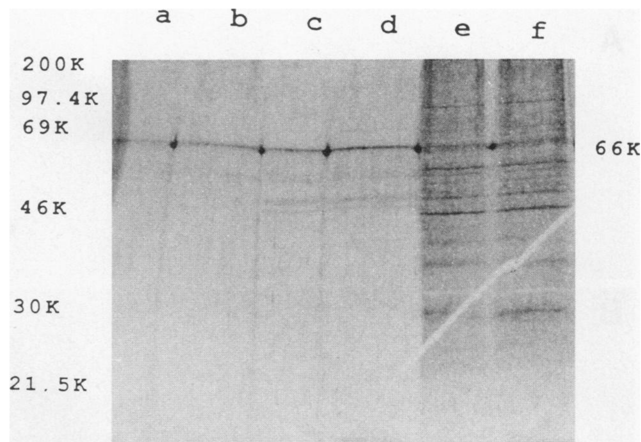

FIG. 2. SDS-PAGE analyses of *C. albicans* fimbriae. A 12.5% acrylamide cross-linked protein minigel was used. The following fimbrial preparations were loaded into the wells of the gel: lanes a and b, 5 μg of EF; lanes c and d, 5 μg of semienriched fimbriae; and lanes e and f, 10 μg of CF. Gel electrophoresis was carried out with a Mini-Protean vertical electrophoresis cell (Bio-Rad) under a constant voltage of 200 V for 50 min. Silver staining technique was used to visualize protein bands.

Inhibition of *C. albicans* adherence to BECs with purified fimbriae. *C. albicans* cells were radiolabelled as previously described by McEachran and Irvin (35) and Smith et al. (48). A loopful of culture from SAB agar (GIBCO) was used as a source of inoculum for 10 ml of M9 medium supplemented with 0.4% (wt/vol) glucose. Cultures were incubated at 25°C for 12 h with 150-rpm agitation in a G25 Gyrotory shaker (New Brunswick Scientific Co.). Cultures were supplemented with 5 μCi of L-[^{35}S]methionine (New England Nuclear, Boston, Mass.) after 10 h of incubation. Cells were harvested by centrifugation (12,000 × *g* for 10 min) and washed three times with 10 ml of PBS, pH 7.2, to remove unincorporated methionine. Washed cells were resuspended in PBS. No clumping was observed during the assay. The amount of L-[^{35}S]methionine incorporated by the *C. albicans* cells was determined by filtering 1.0 ml of a 1:100 dilution of washed *C. albicans* culture through a 0.2-μm-pore-size polycarbonate filter (Nucleopore Corp., Pleasanton, Calif.) in triplicate, washing with 15 ml of PBS, and placing the filter in scintillation vials with 5.0 ml of Aquasol (New England Nuclear). The counts per minute were determined with a Beckman LS-150 liquid scintillation counter. The specific activity of ^{35}S-*C. albicans* cells was generally 0.2 cpm/CFU, and this remained stably associated with the *C. albicans* cells throughout the assay.

BECs (0.5 ml) were preincubated with EF at various concentrations (from 0 to 18 μg of protein per ml) in polystyrene tubes at 37°C for 1 h (final concentrations: 2.0×10^5 BECs per ml). An equal volume of radiolabelled yeast suspended in PBS, pH 7.2, was added to the BECs and incubated at 37°C for 2 h with shaking at 300 rpm. Triplicate aliquots were removed after the assay and filtered through 12-μm-pore-size polycarbonate filters pretreated with 3% (wt/vol) BSA in PBS. BECs were washed with 15 ml of PBS. The filters were then placed in scintillation vials, and the counts per minute were determined as described above. Yeast binding to BECs was corrected for nonspecific binding of yeast to the 12.0-μm-pore-size filter (nonspecific binding was generally less than 15% of the experimental value). The BEC concentration was determined at the end of the assay to correct for cells lost during incubation.

Total and viable cell counts were performed before and after the adhesion assay. Total cell counts were determined with a hemocytometer. Viable counts were determined by serially diluting *C. albicans* in PBS, pH 7.2, and plating appropriate dilutions on SAB agar which were incubated at 37°C until visible and countable colonies formed (usually 24 to 48 h).

RESULTS

Purification of fimbriae from *C. albicans* 40. A typical fimbrial preparation from 200 g (wet weight) of *C. albicans* yielded about 70 mg of CF, which then yielded 5 mg of EF. Fimbriae were removed from washed cells by shearing, separated from cells by centrifugation and filtration, and then subjected to SEC-HPLC. The fimbriae eluted as two peaks (the first peak eluting at the void volume of the column) (Fig. 1). Fimbriae were associated mainly with the first peak, as determined by SDS-PAGE (Fig. 2) and electron microscopy (Fig. 3). The first peak was collected and rechromatographed under identical conditions, with the fimbriae eluting at the void volume of the column (Fig. 1). The second peak on the rechromatography profile indicated that some of the fimbrial preparations were depolymerized and/or deglycosylated during the purification process.

The protein profile of the CF and EF preparations obtained by SDS-PAGE indicated that the molecular mass of the

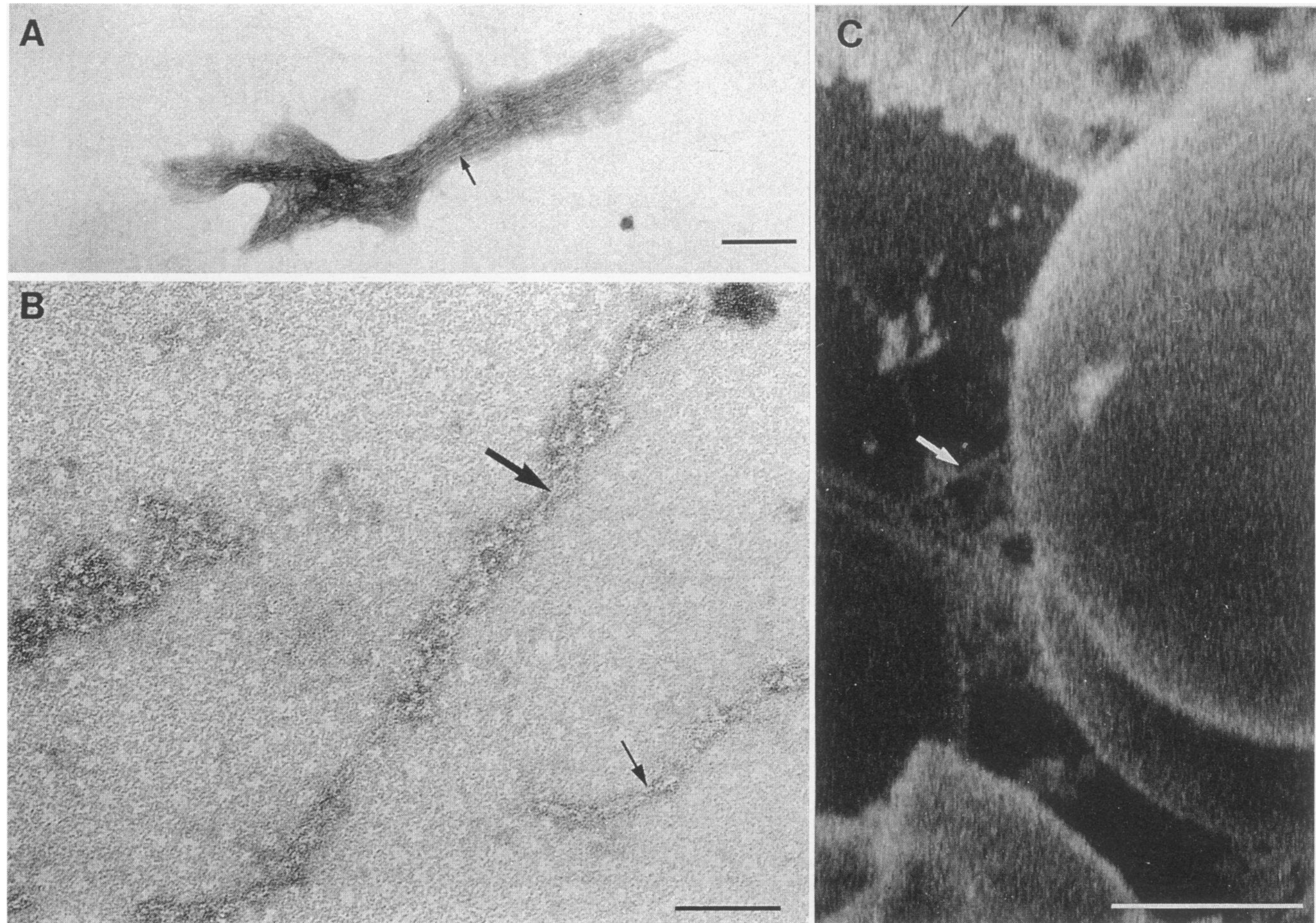

FIG. 3. (A and B) Negatively stained electron micrograph of *C. albicans* EF (arrows). (A) The fimbriae are frequently decorated with material and appear to be flexible; (B) fimbriae frequently aggregated to form loosely associated bundles of filaments. (C) Scanning electron micrograph of *C. albicans* fimbriae. Glutaraldehyde-fixed *C. albicans* [the arrow indicates fimbria involved in *C. albicans* binding to BE(s)] bound to human BECs was postfixed with 2% (wt/vol) osmonium tetroxide in 0.1 M phosphate buffer (pH 7.3) and 1.0% (wt/vol) aqueous tannic acid, critical point dried, and then salted onto a scanning electron microscope stub and examined in a Hitachi S4000 field emission scanning electron microscope operating with an accelerating potential of 2.5 kV. The bars represent 1 μm.

fimbrial subunit was approximately 66 kDa (verified by Western blot analysis; see below). The EF preparation contained almost pure fimbriae (Fig. 2). The 66-kDa proteins were electroeluted from the gel and subsequently separated by reversed-phase HPLC. The main peak was eluted 30 to 34 min after injection onto the column (Fig. 4).

The data from the agglutination assays using anti-*C. albicans* monoclonal antibody Fm16 and polyclonal mouse anti-EF antiserum demonstrated that fimbriae were present on the surface of the cells (Table 1). A negative control using normal mouse IgG failed to agglutinate *C. albicans* yeast. When the negatively stained EF preparations were examined by transmission electron microscopy, large numbers of fimbrial structures ~8 nm in diameter that mediated *C. albicans* binding to BECs were observed (Fig. 3). The fimbriae appeared as flexible filaments that frequently aggregated into small bundles of fimbriae. The fimbriae protruding from the surface of the yeast as filamentous structures appeared to be sparsely distributed but appeared to mediate binding to BECs.

The composition of the fimbrial subunit. The EF preparation was used to determine the protein and carbohydrate composition of the *C. albicans* fimbrial subunit. This was achieved by employing a combination of techniques, including phenol-sulfuric carbohydrate assay, bicinchoninic acid protein assay, and amino acid analysis. The fimbrial subunit was observed to consist of ~85% carbohydrate and ~15% protein. Gas chromatography of the EF sample indicates that the main carbohydrate moiety of the fimbriae consisted of D-mannose. A number of additional minor components in the EF were not identified (data not shown).

The amino acid composition of the fimbrial subunit is shown in Table 2. This fimbrial subunit corresponded to the 66-kDa protein that had been separated by SDS-PAGE and purified by reversed-phase HPLC. On the basis of compositional analysis, an approximate molecular mass of 8,644 Da was calculated for the protein portion of a fimbrial subunit composed of 79 amino acid residues. These results are consistent with the quantitative assessment of carbohydrate and protein in the fimbriae, as the protein component constitutes ~15% of the fimbrial mass.

Antifimbria antibodies. EF were utilized to immunize mice and to produce monoclonal antibodies specific to the fimbriae. ELISA indicated that mouse anti-EF monoclonal antibody Fm16 and polyclonal antibody (sera obtained during the immunization of the BALB/c mice) had high titers for *C.*

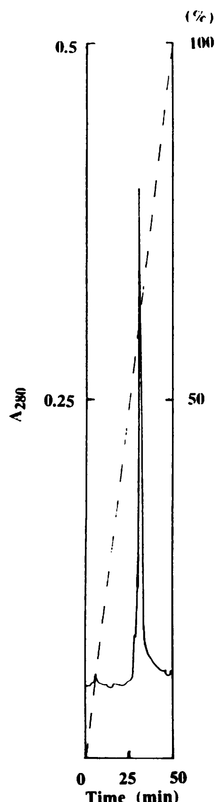

FIG. 4. Reversed-phase HPLC of electroeluted fimbrial protein. CF were subjected to SDS-PAGE, and the fimbrial band on the gel was removed by electroelution. Lyophilized fimbriae were further purified by reversed-phase HPLC on an Aquapore C_4 column (100 by 4.6 mm) with a 7-μm pore size (Pierce) with a linear AB gradient of 2% B per min (where solvent A is 0.05% aqueous trifluoroacetic acid and solvent B is 0.05% trifluoroacetic acid in acetonitrile) at a flow rate of 1 ml/min.

albicans fimbriae (10^6 and 10^5, respectively). Western blot analysis indicated that Fm16 recognized a 66- and a 69-kDa band of fimbrial subunit protein (Fig. 5). The antigenic epitope recognized by Fm16 is a linear peptide sequence (24). Rabbit polyclonal anti-*U. violacea* antiserum specific for *U. violacea* fimbriae (15) was observed to react with the same proteins (data not shown).

The role of *C. albicans* fimbriae in adherence. The role of fimbriae in the mediation of *C. albicans* binding to human BECs was examined. A whole-cell ELISA was performed to assess *C. albicans* fimbria binding to BECs. The data obtained showed that *C. albicans* fimbriae bound to BECs in a saturable and concentration-dependent manner. The binding had almost approached saturation at 50 μg of protein per ml of fimbriae (Fig. 6). The concentration of fimbriae required to reach

TABLE 1. Agglutination of *C. albicans* yeast by anti-*C. albicans* monoclonal antibody Fm16 and polyclonal anti-EF antiserum

Dilution	Agglutination[a] by:	
	Fm16	Anti-EF antiserum
Control (PBS)	−	−
1:1	+++	+++
1:2	++++	++++
1:4	+++	+++
1:8	++	++

[a] Assessed qualitatively by phase-contrast microscopy and reported on a scale from − (no agglutination) to ++++ (heavy agglutination). Normal mouse IgG showed no agglutination with any of the dilutions tested.

TABLE 2. Amino acid composition of fimbrial subunits from *C. albicans* 40

Amino acid residue	No. of residues[a] (integer value)/fimbrial subunit
Asx	8.24 (8)
Thr[b]	4.73 (5)
Ser[b]	5.73 (6)
Glx	8.17 (8)
Pro	2.79 (3)
Gly	7.02 (7)
Ala	6.00 (6)
Cys	ND
Val	5.57 (6)
Met	0.82 (1)
Ile	4.73 (5)
Leu	6.82 (7)
Tyr	2.55 (3)
Phe	3.21 (3)
His	1.48 (1)
Lys	5.91 (6)
Trp	ND
Arg	3.81 (4)

[a] Determined experimentally. ND, not determined. The total of 79 residues had an estimated molecular weight of 8,644.

[b] No correction was made for half-Cys and Trp or for the destructive loss of Ser and Thr during hydrolysis.

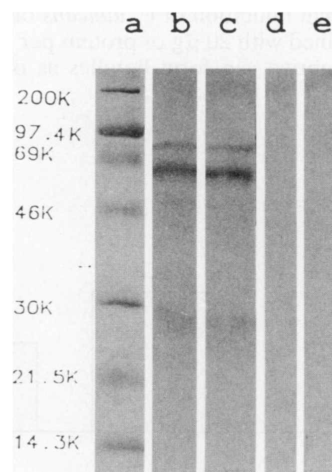

FIG. 5. Western blot analysis of *C. albicans* EF. Fimbriae were loaded into the wells (30 μg per well) in a 12.5% acrylamide cross-linked running gel, separated by SDS-PAGE, and transblotted onto nitrocellulose membranes as described in Methods and Materials. Lane a shows the molecular weight markers. Fimbriae in lanes b and c were probed with murine ascitic fluid containing antifimbria monoclonal antibody Fm16 (diluted 1:500), and lanes d and e were probed with an affinity-purified commercial normal mouse IgG (diluted 1:500). A goat anti-mouse IgG–alkaline phosphatase conjugate was used as the secondary antibody. The bands were obtained with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as the alkaline phosphatase substrates.

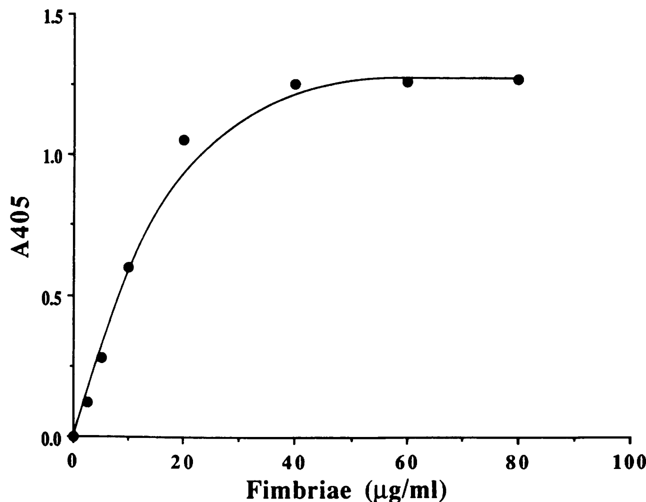

FIG. 6. Direct binding of *C. albicans* fimbriae to human BECs. A whole-cell ELISA was employed to assay for the adherence of fimbriae to BECs (collected from 10 healthy, nonsmoking male volunteers). *C. albicans* EF were incubated with BECs for 1.5 h at room temperature in PBS, pH 7.2. A murine anti-*C. albicans* fimbrial monoclonal antibody, Fm16, and an anti-mouse IgG–horseradish peroxidase conjugate were employed to quantitate EF binding to BECs which were collected on the surface of 12-µm-pore-size polycarbonate filters (Nucleopore). The binding of fimbriae to BECs was represented as the A_{405} observed in relation to the concentration of fimbriae used.

half-maximal binding was determined to be 10 µg/ml, indicating that the binding to cell surface receptors is of high avidity.

Preincubation of BECs with purified fimbriae before the addition of *C. albicans* yeast resulted in the inhibition of whole-cell binding to BECs. The fimbria-mediated inhibition of *C. albicans* binding to BECs was concentration dependent (Fig. 7). A maximal inhibition of *C. albicans* binding to BECs of 64% was obtained with 20 µg of protein per ml of fimbriae. Although the fimbriae can form bundles as observed in the

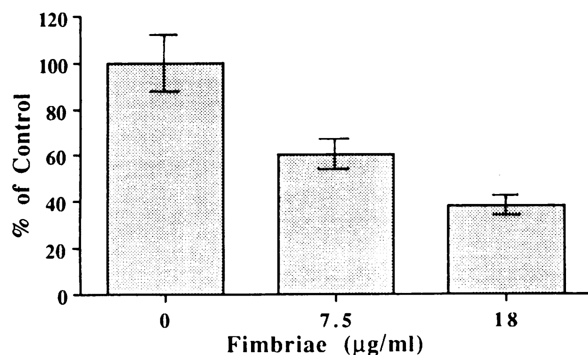

FIG. 7. Inhibition of *C. albicans* whole-cell binding to human BECs by direct competition with EF. The bars represent ±1 standard deviation. The adherence assay of Staddon et al. (49) which involved metabolically ^{35}S-labelled *C. albicans* was used. BECs (2×10^5 cells per ml of PBS, pH 7.2) were preincubated with various concentrations of EF (0 to 20 µg of protein per ml) for 1 h at 37°C prior to their addition to radiolabelled yeast (ratio of 100 yeast cells to 1 BEC). The assay mixture was incubated for 2 h at 37°C, shaking at 300 rpm on a Gyrotory shaker. Washed BECs with adherent *C. albicans* were transferred to glass scintillation vials, and the counts per minute were recorded.

electron micrographs, they did not cause any problems with our direct binding or binding inhibition assays.

DISCUSSION

C. albicans has been found to bind to human BECs (8, 11, 28, 29, 49), but the adhesins which *C. albicans* utilizes to bind to BECs have not been purified and characterized. *C. albicans* possesses fimbriae which are morphologically analogous to bacterial fimbriae (16). Although several studies have suggested that fibrous structures, perhaps fimbriae, mediate the adherence of *C. albicans* to BECs (12, 27, 33, 34, 36, 37, 52), these fimbriae have not been well characterized. This may be due, in part, to the difficulty of purifying the fimbriae. We have described the isolation and purification of *C. albicans* fimbriae and demonstrated that fimbriae can mediate adherence of these organisms to human BECs.

Fimbriae were easily sheared from the cell surfaces of *C. albicans* when subjected to gentle shearing by homogenization. The purification of fimbriae from the crude extract was achieved by two successive rounds of SEC-HPLC. *C. albicans* fimbriae were not very stable and were readily degraded or depolymerized during the purification processes. Optimal preservation of fimbriae was achieved by maintaining the samples on ice and by utilizing phenylmethylsulfonyl fluoride as a protease inhibitor. The addition of calcium appeared to stabilize the fimbrial structure and minimize depolymerization. This is in agreement with the results reported by Gardiner and Day which indicated that calcium is important in maintaining the structural integrity of fungal fimbriae (16).

Electron microscopic analysis revealed the appearance of long filaments in the EF preparation. SDS-PAGE analysis of CF and EF preparations revealed a major 66-kDa band along with a few faint lower-molecular-mass bands. Successive SEC-HPLC removed most of the minor components from the CF preparation, resulting in a purer EF preparation (Fig. 2). Western blot analysis indicated the presence of two major bands (66 and 69 kDa) which were detected with monoclonal antibody Fm16, which can agglutinate *C. albicans* (Table 1). The two major bands that were recognized by Fm16 may represent fimbrial subunits with different degrees of glycosylation. The minor component of 28 kDa (and occasionally a 9-kDa protein) may be a degraded product of the major bands or unglycosylated fimbrial subunits.

Carbohydrate and protein analyses indicated that the fimbrial subunit was composed of approximately 85% carbohydrate and 15% protein by weight. The gas chromatogram of the EF sample revealed that the main component in the carbohydrate moiety of the fimbrial subunit consisted of D-mannose. Our findings differ from those of Gardiner and Day (16) in that purified fimbriae from *U. violacea* did not contain any detectable carbohydrate component. Subsequent studies by Castle et al. (5) demonstrated that *U. violacea* fimbriae contained approximately 10% carbohydrate (mannose). Unlike *U. violacea* fimbriae, the *C. albicans* fimbrial subunit is normally extensively glycosylated with mannose residues. *C. albicans* yeast forms appeared to be sparsely fimbriated (Fig. 3), whereas *U. violacea* has numerous fimbriae protruding from its cell surface (40).

Amino acid analysis of the *C. albicans* fimbrial subunit indicated that the most frequent amino acid residues of the protein portion of fimbriae were Val, Asx, Glx, Ser, Thr, Gly, Leu, Ile, Lys, and Ala, while little methionine or histidine was detected. The fimbrial subunit has a high proportion of hydrophobic residues (50% of the total residues) and a reasonably high level of basic amino acid residues (12.5% of the total

residues). The *C. albicans* fimbrial subunit has an amino acid composition that is proportionally similar to that reported for the *U. violacea* fimbrial subunit, which has a molecular mass of 74 kDa (9). The hydrophobicity of the *C. albicans* fimbrial subunit (50% of the total residues) is slightly higher than that of the *U. violacea* fimbrial subunit (35% of the total residues). Amino acid analysis of the *C. albicans* fimbrial subunit also indicated that the molecular weight of the protein portion of fimbrial subunit was 8,644, based on a compositional analysis assuming 79 amino acid residues (~15% of the mass of the fimbrial subunit). This is consistent with the results obtained by both carbohydrate and protein analyses. The fimbrial subunit has an apparent M_r of approximate 66,000 as determined by SDS-PAGE (Fig. 2), slightly lower than the M_r of the *U. violacea* fimbrial subunit. N-terminal amino acid sequencing of the *C. albicans* fimbrial subunit was unsuccessful, leading us to believe that the N-terminal residue was blocked. The nature of the blocking group is unknown at this time. In summary, the major structural subunit of the fimbriae is a glycoprotein which consists of 80 to 85% carbohydrate (primarily D-mannose) and 10 to 15% protein.

Elucidation of the mechanisms which mediate adhesion may allow new therapies to be developed to prevent or treat infection. The role of *C. albicans* fimbriae in the adherence of *C. albicans* to BECs was examined in this paper. First, the direct binding assay indicated that the *C. albicans* fimbriae do bind to BECs. The binding of *C. albicans* fimbriae to BECs was concentration dependent and saturable at ~50 μg of protein per ml of EF (Fig. 6). Second, inhibition assays showed that *C. albicans* fimbriae significantly inhibited the binding of *C. albicans* whole cells to BECs. This implied that the interactions between fimbriae and BECs were specific and involved cell surface receptors that can recognize and bind to the *C. albicans* fimbrial adhesins. These results indicated that the *C. albicans* fimbriae are functional adhesins. In the case of the *U. violacea*, fimbriae may have a role in conjugation (41).

Equilibrium analysis of binding has indicated that the adherence of *C. albicans* to BECs involves multiple adhesin-receptor interactions (49). Several cell surface molecules have been identified as possible adhesins, such as CR3-like and β1-integrin like molecules of *C. albicans* (32), lectin-like molecules of *C. albicans* (11, 12, 50), the factor 6 moiety of *C. albicans* (14, 38), and the secreted acid proteinase of *C. albicans* (1, 43, 44). Thus far, none of them have been extensively characterized.

We have demonstrated that *C. albicans* fimbrial adhesins mediate the binding of *C. albicans* to BECs in vitro, but the role of *C. albicans* fimbriae in mediating the adherence of *C. albicans* to BECs in vivo is still unclear. While we have identified the major fimbrial subunit, it is unclear whether there are additional minor components that contribute to the fimbrial structure and function. The role of these fimbriae in pathogenesis remains to be resolved.

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