Scanning Electron Microscopy of Epidermal Adherence and Cavitation in Murine Candidiasis: a Role for *Candida* Acid Proteinase

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Adherence of blastoconidia to epidermal corneocytes is an early event in *Candida* colonization and infection of the skin. Pathogenic species adhere more avidly than nonpathogenic species, transform to hyphal growth, and invade the stratum corneum of the skin. Adherence was studied by scanning electron microscopy of experimental murine cutaneous *Candida* infections, using six species of *Candida*. *Candida albicans* and *C. stellatoidea* blastoconidia, applied to newborn mouse skin, adhered to the stratum corneum in greater numbers than other species tested, acquired fibrils and strands of amorphous mucinlike material ("cohesin") between spores and the corneocyte cell surface, formed cavitations in the corneocyte surface, and invaded the corneocyte envelope by hyphal growth at sites distant to the point of blastoconidia attachment. Other species showed little or no adherence, colonization, or cavitation of the corneocyte surface, except *C. tropicalis*, which showed intermediate results. Pepstatin, an inhibitor of *Candida* acid proteinase, did not alter adherence or cohesion formation, but inhibited formation of corneocyte cavitations about adherent blastoconidia, suggesting that this enzyme may facilitate adherence/invasion events on skin. Depletion of surface lipids did not alter the formation of cohesin material or the adherence process. Adherence and invasion of epithelium by pathogenic *Candida* species include the interaction of blastoconidia with an epithelial surface cohesin material that participates in the adherence process. *Candida* acid proteinase, a keratinolytic enzyme, may participate in the cavitation process of the corneocyte surface by *C. albicans*.

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*Candida albicans* is a dimorphic fungus that commensally colonizes the cutaneous and mucosal surfaces of humans. In settings in which the epithelial barrier or host immunity or both are altered or suppressed, *C. albicans* and occasionally other related species produce opportunistic infections of the skin and mucosal cavities. Initial events of cutaneous candidiasis include adherence of blastoconidia to epithelial cell surfaces, fungal proliferation and colonization, and invasion of epithelial tissue. In vitro studies show that adherence to corneocytes (25) and mucosal cells (8) is associated with pathogenic species. The same species are capable of hyphal invasion of corneocytes and stratum corneum in rodent models of this infection (22, 24). Hyphal transformation of *Candida* precedes invasion by the organism into the epidermis, which is met by a complement-dependent neutrophilic inflammatory response centered about invading hyphae (21, 23). This response is protective and confines the infection to the superficial epidermis. Nonpathogenic species adhere poorly to corneocytes or mucosal cells (8, 21) and do not invade the stratum corneum in animal models (22, 24).

To characterize initial adherence and invasion steps in experimental murine cutaneous candidiasis, we examined six species of *Candida* applied under occlusion to newborn mouse skin in vivo by scanning electron microscopy. *Candida* acid proteinase (CAP), a skin keratin-cleaving extracellular enzyme (14, 18, 25, 27; T. L. Ray, and C. D. Payne, Clin. Res. 30:801A, 1982), is a putative virulence factor (11). To evaluate the participation of CAP during early invasion, we studied the effect of pepstatin, a known inhibitor of CAP (18, 27), on adherence and invasion.

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**MATERIALS AND METHODS**

**Cultures.** One strain each of *C. albicans* (serotype A), *C. stellatoidea*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii*, and *C. krusei* was obtained from clinical isolates and identified by sugar assimilation and fermentation criteria (31); these were the same strains used in previous adherence studies (21). They were maintained on Sabouraud dextrose (S-D) agar slants or plates at room temperature. *Candida* blastoconidia for experimental infections were grown in S-D broth for 24 to 48 h at room temperature, harvested by centrifugation (1,500 × g), and washed twice in sterile 0.1 M sodium phosphate-0.85% sodium chloride buffer (phosphate-buffered saline), pH 7.0. Blastocandida were suspended in fresh S-D broth at a concentration of 10⁷ blastocandida per ml as determined by hemacytometer counts. Cell viability was >99% by trypan blue dye (0.08%) exclusion criteria.

**Controls.** Dead *C. albicans* blastoconidia were produced by (i) autoclaving S-D broth cultures at 121°C and 15 lb/in² for 20 min (heat killed) and (ii) suspending *C. albicans* cells in 10% neutral buffered Formalin for 30 min (Formalin killed). Both preparations were washed twice in phosphate-buffered saline and suspended in fresh S-D broth prior to use. Culture sterility was confirmed by trypan blue exclusion and viable counts on S-D agar. A comparably sized latex bead (5.7 μm) (Sigma Chemical Co., St. Louis, Mo.) was used as a control particle, suspended in S-D broth at a concentration of 10⁹ beads per ml. All were applied to mice in the same manner as live *Candida* blastoconidia. As an inert surface control, *C. albicans* in S-D broth was grown for up to 48 h on 0.2-μm-pore size nylon filters (Nylon-66 filters; Rainin Instrument Co., Woburn, Mass.) placed on S-D agar.
Filters and control specimens were processed in the same manner as test specimens, detailed below.

**Experimental infections in newborn mice.** Swiss Webster mice, 1 to 2 days old, were inoculated epicutaneously with *Candida* cultures or controls by previously described methods (22). A 4-mm² nonwoven cotton patch (Webril; Kendall, Chicago, Ill.), saturated with a 10-μl sample of the *Candida* test suspension (10⁷ blastoconidia), was placed on the backs of animals and fixed with occlusive tape dressing (Blenderm; 3M, St. Paul, Minn.). After intervals of 0.5, 4, 6, 8, 12, 24, and 48 h, animals were sacrificed, the patches were removed, and skin specimens (4 by 8 mm) were obtained from the inoculation sites.

**Scanning electron microscopy.** Specimens were fixed overnight in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.25, at 4°C. Specimens were then washed in three changes of buffer for 1 h and postfixed in 2% osmium tetroxide in buffer for 1 h. After a 30-min wash in two changes of buffer and a 1-min wash in distilled water, samples were dehydrated in graded concentrations of acetone or ethanol (50 to 100%) and critical-point dried in 100% CO₂ (Samdri 790; Toussim Research Corp., Rockville, Md.). Specimens, mounted on carbon stubs with colloidal graphite, were coated with gold-palladium in a low-pressure argon atmosphere with a sputter coater (Technics, Alexandria, Va.). Tissue was viewed on a JEOL JSM 35C scanning electron microscope at an accelerating voltage of 13 kV.

To control for artifact, fresh specimens were submerged in liquid propane for 5 s and then kept in liquid nitrogen until lyophilized at −50°C and 50 mtorr for 96 h. Specimens were then mounted on carbon stubs with colloidal graphite, carbon-coated (vacuum evaporator type HUS-4; Hitachi Ltd., Tokyo, Japan), and viewed as above.

**Transmission electron microscopy.** Skin tissue for transmission electron microscopy was fixed overnight in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.25, at 4°C. After being rinsed for 5 min in fresh buffer, the samples were postfixed in 1% osmium tetroxide in buffer for 2 h at room temperature. After a wash in distilled water for 30 min with four changes, the tissue was placed in 2% uranyl acetate for 2 h and washed four times in distilled water. Dehydration was performed in 70% acetone for 30 min and 100% acetone for 30 min. Tissue was embedded in 50% Spurr resin in acetone for 12 h, followed by 100% resin for 24 h. Specimens were then placed in flat block molds containing fresh resin and polymerized for 48 h in a desiccator at 70°C.

Thin sections, cut on an LKB Ultrotome V (LKB Instruments, Inc., Rockville, Md.) and placed on acid-etched copper grids and stained with uranyl acetate and lead citrate (26), were examined on a JEOL 100B or Hitachi H-600 transmission electron microscope at 60 kV.

**Pepstatin inhibition of CAP.** To assess the role of CAP released by blastoconidia, this enzyme was inhibited by the addition of pepstatin (Sigma) at a concentration of 2.5 μg/ml to test suspensions of viable *C. albicans* blastoconidia. Pepstatin has been shown previously to inhibit this enzyme (18, 27; Ray and Payne, Clin. Res. 30:801A, 1982), and the concentration used was a fivefold excess of pepstatin needed to completely abolish enzyme activity in 5-day culture supernatants of *C. albicans*.

**Depletion of skin surface lipid.** To assess the participation of surface lipids in adherence, the backs of newborn mice were extensively washed with chloroform-methanol (2:1, vol/vol), a standard skin lipid extraction solvent, by wiping 10 times with a saturated cotton gauze sponge to remove surface lipid. Animals were inoculated immediately thereafter with test suspensions of *C. albicans*.

**RESULTS**

**Scanning electron microscopy.** (i) **Adherence of blastoconidia.** *C. albicans* and *C. stellatoidea* blastoconidia adhered to newborn murine epidermis in greater numbers than the other species at every time point studied, in agreement with in vitro studies of human corneocyte adherence by *Candida* species (21). *C. albicans* and *C. stellatoidea* blastoconidia were readily apparent, easily found in low-power fields, and totaled 10⁴ to 10⁵ blastoconidia per specimen at 4 h, and their numbers increased at each time point. The other species were found only after extensive searching and were few in number (50 to 100 blastoconidia per specimen) at each time point.

Adherent *C. albicans* and *C. stellatoidea* blastoconidia were located within crevices of corneocyte cell junctions and on the open surface of cells (Fig. 1). Most were coated with strands or sheets of amorphous mucinlike material linking adjacent blastoconidia and blastoconidia with the corneocyte surface (Fig. 2). This material had a "stretched," strandlike appearance and was tentatively called "cohesin." Cohesin adhered to most, but not all, blastoconidia and variably spared or adhered to newly formed buds of proliferating spores (Fig. 3B). Similar material was noted as a filmlike covering on the corneocyte surface, distant from sites of blastoconidia attachment (Fig. 3A). Cohesin was present at 30 min and later after application. Mycelial elements also acquired cohesin along their margins, although it was not as abundant as that about adherent spores. Cohesin was minimally associated with *C. tropicalis* and *C. parapsilosis* blastoconidia and was not seen with *C. guilliermondii* and *C. kruzei*, species that exhibit little or no adherence for corneocytes (Table 1).

(ii) **Colonization.** From 8 to 24 h, colonization of the skin surface by *C. albicans* (Fig. 4) and *C. stellatoidea* and, to a much lesser degree, *C. tropicalis* and *C. parapsilosis* was evident. Clusters of spores, budding spores, and mycelial elements formed colonies of growth over all aspects of the skin surface, including sites of erupting hair follicles. Myce-
lial growth included short germ tube formation and long mycelial forms that coursed over the surface in an apparent-ly random manner. Colony size, number of organisms, and proportion of mycelial elements increased with time. Other species tested showed little proliferation or colony formation over time and typically did not develop mycelial elements, though some were found.

(iii) Mycelial Invasion. Mycelial elements of *C. albicans* and *C. stellatoidea* penetrated the corneocyte surface (Fig. 5). Invasion was typically at sites distant to blastoconidial attachment and occurred at the acral tip of mycelia. Mycelial invasion of the skin occurred on both open and convoluted cell surfaces and at corneocyte cell junctions. Minimal disruption of the surface was noted at invasion sites, with only slight depressions confined to a 1-μm perimeter about invading mycelia. Other species did not invade.

(iv) Cavitation. Depressions were noted in the corneocyte surface about adherent blastoconidia 4 h after application of *C. albicans* (Fig. 6A), *C. stellatoidea*, and, to a lesser degree, *C. tropicalis*, but not other species tested. By 8 h, adherent spores appeared embedded in the corneocyte surface, but were not incorporated into the cell. Similar features were observed along the margins of mycelial elements. These depressions suggested an active cavitation process affecting the corneocyte surface, as they were not observed with killed blastoconidia or latex beads, and did not appear to be artifact. Spores applied in S-D or phosphate-buffered saline and specimens dehydrated in either acetone or ethanol still exhibited cavitations. Specimens frozen in liquid nitrogen, freeze-dried, and carbon coated still retained cavitations. Similarly, cohesin was unaltered by any of the tissue-processing techniques (data not shown). When adherent blastoconidia had become dislodged, cavitations remained, leaving an "egg carton" dimpling of the corneocyte surface (Fig. 6B). The above features are summarized for each of the *Candida* species in Table 1.

(v) Controls. Snap-frozen, lyophilized, carbon-coated specimens exhibited the same features as glutaraldehyde-fixed, gold-palladium-coated specimens, including cohesin and cavitation, in appropriate species. Only the latter are presented in the figures. Formalin- or heat-killed blastoconidia and 5-μm latex beads adhered poorly to corneocytes in vivo and were found in very small numbers (<25) after the entire specimen surface was examined. They did not acquire cohesin strands or coating and did not cause cavitation of the surface (Fig. 7).

*C. albicans* grown on nylon filters retained their blastocoidal morphology without hyphal transformation. No surface material resembling cohesin was found at any time up to 48 h (data not shown).
TABLE 1. Properties of Candida species

<table>
<thead>
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<th>Adherence</th>
<th>Cohesin binding</th>
<th>Cavitation</th>
<th>Budding</th>
<th>Mycelia</th>
<th>Colonies</th>
<th>Invasion</th>
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* +++ , Marked; ++, moderate; +, minimal; -, absent.

b Reference 21.
c Reference 22.
e Invasive if inoculated below the stratum corneum (24).

(vi) Skin lipid deletion. C. albicans applied to mouse skin previously depleted of surface lipid by repeated wiping with CHCl-MeOH (2:1) adhered to corneocytes and acquired cohesin strands and sheets that were identical to those of untreated skin. Proliferation, budding, and colonization were unaffected by lipid depletion of the skin (data not shown).

(vii) Pepstatin inhibition of CAP. The addition of 2.5 μg of pepstatin per ml to C. albicans culture inocula, to inhibit the exoenzyme CAP, did not alter the number of adherent blastoconidia or the binding of cohesin to these organisms. However, cavitation was absent about blastoconidia and mycelia for up to 16 h (Fig. 8) and was minimally present at later time points. Invading mycelia were not encountered in pepstatin-treated specimens.

Transmission electron microscopy. Transmission electron microscopy of 24-h infections revealed Candida elements in the stratum corneum. A characteristic electron-dense, broad Candida cell wall was contiguous with strands of material bridging to the adjacent corneocyte envelope and cell membrane. This was morphologically identical to the ruthenium red-positive matrix seen in oral and urinary mucosa (16) and buccal and vaginal mucosa (1) studies and may represent the cohesin visualized by scanning electron microscopy (data not shown).

DISCUSSION

Adherence of Candida species to mucosal and cutaneous epithelial cells is an initial event in the colonization and infection of oral, vaginal, and skin surfaces (6-8, 12, 21, 32). In vitro studies demonstrate that pathogenic species of Candida (C. albicans and C. stellatoidea) bind to epithelial cells more avidly than nonpathogenic species (8, 21), which may contribute to their prevalence as commensal and opportunistic organisms. Adherence may involve lectinlike inter-
actions of Candida cell wall peptidoglycans with epithelial cell surface factors shared by mucosal and skin keratinocytes. Candida adherence to mucosal and skin epithelium appears to be similar (21). Specific Candida cell wall moieties responsible for the adherence phenomenon are not well characterized. Mannose and fucose sugars of the cell wall carbohydrates (peptidoglycans) have been proposed; however, some studies suggest that mannans is not solely responsible for adherence (12, 21, 29, 30, 32). Chitin, another cell wall constituent, may also participate (13). Current evidence suggests that the Candida moiety involved in adherence is a cell wall glycoprotein that is variably susceptible to α-mannosidase and protease degradation. The protein portion may be essential for its function (2, 4, 17). Adherence to inert surfaces involves attractive London-van der Waals forces (hydrophobic forces [10]), and a charge phenomenon may also participate in Candida adherence to epithelial surfaces.

The murine in vivo studies reported here are similar to in vitro studies showing that C. albicans and C. stellatoidea readily adhere to human epidermal corneocytes. Both species are pathogens in experimental rodent cutaneous candidiasis (22, 24). Other Candida species tested exhibited minor or negligible adherence and are nonpathogens in skin models (Table 1). Adherence of viable blastoconidia is significantly greater than dead blastoconidia (21). Either the process requires a metabolically active mechanism, or the Candida adherence factors are altered or destroyed during Candida killing. Both heat and irradiation with UV light release carbohydrate complexes from C. albicans, which inhibit epithelial cell adherence (12) and neutrophil attachment (3). Cell wall peptidoglycomannans may be susceptible to both Formalin and heat and account for the lack of adherence and cohesin in controls (Fig. 7). Adherence appears to be specific, since latex beads of comparable size were neither bound nor trapped by the murine skin surface and did not acquire cohesin.

Ultrastructural visualization of adherence demonstrated amorphous mucinlike material coating blastoconidia of some, but not all, Candida species. We call this material cohesin, since it morphologically differs from bacterial "adhesin" polymers and appears to be derived, at least in part, from corneocytes. Cohesin was found as a film of loosely adherent strandlike material on the corneocyte surface of newborn mouse skin, both in association with and independent of adherent blastoconidia. It was not altered or removed by lipid-extracting solvent. It seems unlikely that cohesin is an artifact, since it was found only with viable C. albicans, C. stellatoidea, and C. tropicalis adhering to skin and not with the other species tested. It was not found in Candida blastoconidia cultures similarly applied to nylon filter surfaces. It was present when either acetone or ethanol dehydration of tissue was used and appears identical in specimens that are either glutaraldehyde fixed, critical-point dried, and gold-palladium coated or snap-frozen, lyophilized, and carbon coated. It was not seen in killed Candida or latex particle control studies, indicating that it is not a product of the inoculation process or fixation.

Cohesin binds to blastoconidial and mycelial surfaces, structurally linking Candida to the corneocyte cell surface within 30 min after blastoconidia application. This suggests that cohesin is preformed. Though not chemically defined, it may be a glycolcalyx cuticle on corneocytes, possessing a lectinlike component that participates in Candida adherence. Precedence exists for this concept as similar extracellular "mucilike" material has been noted in scanning electron microscopy studies of Candida adherence to gastrointestinal epithelium (20) and vaginal epithelial cells (19). A ruthenium red-positive, electron-dense matrix has been observed by transmission electron microscopy, linking blastoconidia to vaginal, buccal, and urinary epithelial cells (1, 16), suggesting a mucopolysaccharide component.

Colonization of skin by adherent blastoconidia included the generation of budding blastoconidia, germ tubes, and mycelial elements within 4 to 8 h of inoculation and pro-
ceed to extensive fungal residence by 24 h. Colonies were not restricted to specific or unique regions of the corneocyte, although convolutions, crevices, and follicular openings were commonly colonized, possibly due to trapping as well as adherence.

Invasion of the corneocyte surface by mycelial elements is characteristic of C. albicans and C. stellatoidea, in agreement with experimental murine cutaneous candidiasis studies (22, 24). Acral tips of mycelia penetrate the cell surface at sites distant to blastoconidial attachment, similar to the process described by Howlett and Squier on rat tongue epithelium (5). We did not encounter microridges as seen on vaginal epithelial cells (19) or penetration "through holes in the keratinized cells" as described by Wilborn and Montes (34). The cell surface was not deformed and stress lines were not evident. Whether physical or hydrostatic forces were operative could not be discerned. Most mycelia establish considerable cell surface contact before invading, which may provide a foundation against which physical/mechanical forces effect penetration. Invasion occurred on all surface aspects of the corneocyte, suggesting that the entire cell, and not just cell junctions, is susceptible to invasion.

Cavitations were noted early in the adherence/colonization process as corneocyte surface depressions restricted to the immediate perimeter of adherent blastoconidia and mycelia, which become embedded into the surface. Transmission electron microscopy studies of mucosal and vaginal epithelium report analogous findings, which are attributed to phagocytic mechanisms (1). Similar "burrowing" of C. albicans into vaginal epithelial cells (19) and vascular endothelial cells (9) has been observed. Corneocytes are devoid of intracellular organelles and are metabolically inactive, making endocytosis or phagocytosis of Candida by corneocytes unlikely explanations for cavitation.

Pathogenic Candida species produce CAP, an exoenzyme with aspartic proteinase activity (15, 18, 25, 27). Studies of experimental systemic candidiasis in mice with CAP-deficient and CAP-sufficient C. albicans strains and mutants indicate that this enzyme is a virulence factor (11). CAP digests the major epidermal protein keratin (18) and is mainly produced by C. albicans, C. stellatoidea, and C. tropicalis (14, 28) and specifically the strains of those species used in this study (Ray and Payne, Clin. Res. 30:801A, 1982). Pepstatin inhibits CAP activity (18, 27) and the growth of C. albicans in human stratum corneum-supplemented media (33). Thus, the role of CAP in cavitation was assessed by inhibition with pepstatin. Cavitation was abrogated by pepstatin for up to 16 h and was minimally present at 24 h. The enzyme was optimally active at acid pH (4.0 to 4.5), with negligible neutral pH activity. Conceivably, actively growing...
Cohesin coating is retained, but cavitation is absent when blastoconidia are applied in the presence of 2.5 μg of pepstatin per ml. (B) Control without pepstatin shows cohesin and cavitation about adherent blastoconidia. Critical-point dried; bars, 5 μm each.

Candida cells provide a circumjacent acidic milieu conducive to enzyme activity, restricting cavitation to the perimeter of adherent live organisms. This mechanism might contribute to the "phagocytic vacuoles" and burrowing reported in other epithelia (1, 19) and vascular endothelium (9). CAP may also participate in Candida invasion through stratum corneum, epidermis, and dermis, since enzyme-producing species (C. albicans, C. stellatoidea, and C. tropicalis) are capable of invading the entire cutis under appropriate experimental conditions (24).

Pathogenesis of experimental murine cutaneous candidiasis includes adherence of blastoconidia to the stratum corneum that involves the interaction of Candida cell wall constituents with a cohesin on the corneocyte surface. Colonization and mycelial growth lead to cavitation and mycelial invasion of the corneocyte. CAP, generated by pathogenic species of Candida, may mediate these events and contribute to their cutaneous and epithelial pathogenicity.

ACKNOWLEDGMENT

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LITERATURE CITED