Alterations to the Cell Wall of *Histoplasma capsulatum* Yeasts during Infection of Macrophages or Epithelial Cells

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Many *Histoplasma capsulatum* strains have α-(1,3)-glucan in their cell walls and spontaneously produce variants that lack this polymer. The variants, in contrast to the parents, exist in aberrant shapes within macrophages. Here, the ultrastructure of the parental and variant cell walls was examined. All yeasts had identical electron-lucent, thick walls when grown in broth culture. However, ingestion by either macrophages or hamster trachea epithelial (HTE) cells caused the walls of variants to become electron-dense, thin, and sinuous. Parental strains remained unchanged in macrophages. Within HTE cells inoculated with parental strains, some organisms retained a thick wall and α-(1,3)-glucan but appeared to be degrading. In contrast, apparently intact intracellular yeasts had thin, wavy walls lacking α-(1,3)-glucan. A microenvironment within HTE cells that is unfavorable for the parental phenotype may trigger this ultrastructural change, potentially explaining why only variant yeasts are harvested from such cultures.

Chemotype II strains of *Histoplasma capsulatum*, a fungus that causes respiratory disease in mammals, have α-(1,3)-glucan within the cell walls of the yeast form [1]. These yeasts clump in liquid culture and form rough-textured colonies on solid medium. Chemotype II strains, which include isolates morphologically similar to the parental strain [5], have thin, wavy walls lacking α-(1,3)-glucan. These variants to become electron-dense, thin, and sinuous. Parental strains remained unchanged in macrophages. Within HTE cells inoculated with parental strains, some organisms retained a thick wall and α-(1,3)-glucan but appeared to be degrading. In contrast, apparently intact intracellular yeasts had thin, wavy walls lacking α-(1,3)-glucan. A microenvironment within HTE cells that is unfavorable for the parental phenotype may trigger this ultrastructural change, potentially explaining why only variant yeasts are harvested from such cultures.

Materials and Methods

**Yeast strains.** *H. capsulatum* G186A-R, -S, and -HTE as well as strains UCLA 531-R, -S, and -HTE were acquired and maintained as previously described [4]. The suffix ‘‘R’’ refers to the rough colony phenotype of the parental strain. These parent strains are representative of two separate evolutionary classes [10] within chemotype II. Broth cultures of any strain are routinely >95% viable, as assessed by staining with fluorescein-diacetate and ethidium bromide [11].

**Mammalian cells.** P388D1-D2 cells, a randomly selected clone from P388D1 macrophage-like cells [4], and HTE cells, a non-transformed epithelial line derived from hamster trachea [12], were cultured in Ham’s F-12 (GIBCO Laboratories, Grand Island, NY) completed with 100 U/mL penicillin, 110 μg/mL streptomycin, and 10% fetal bovine serum (HyClone Laboratories, Logan, UT).

**Electron microscopy.** To examine yeasts in suspension, we washed broth-grown organisms twice in PBS and then fixed them for 2 h in cold 2% glutaraldehyde (Polysciences, Warrington, PA) containing 0.15% ruthenium red in modified Tyrode’s buffer, pH 7.4 (see [13]; contains sucrose rather than glucose and 6.0 rather than 8.0 g/mL NaCl). Pelleted yeasts were postfixed at 4°C in 1% osmium tetroxide and 0.15% ruthenium red in the modified...
Tyrode’s buffer, rinsed in buffer, and dehydrated with ethanol and propylene oxide prior to embedding (Poly/Bed 812; Polysciences). Thin sections were cut with a glass knife, mounted on copper grids, and stained with lead and uranyl acetate. Grids were examined using a Phillips 201 electron microscope, and representative fields were photographed.

During experiments to localize α-(1,3)-glucan, ruthenium red was left out of the fixation and postfixation steps. Thin sections were washed in PBS containing 0.5%–1% bovine serum albumin before monoclonal antibody MOPC 104E (Sigma, St. Louis) was applied as a primary antibody for 1 h. This antibody is specific for α-(1,3)-linked oligosaccharides [14] and binds to rough strains but not to smooth variants, which lack α-(1,3)-glucan [15]. After 2 more washes, samples were incubated for 30 min with goat anti–mouse IgM coupled to 15-nm gold particles (Zymed, San Francisco) for 30 min. Samples were then washed and examined for gold particles.

We examined the ultrastructure of yeasts ingested by macrophages by seeding 2.8 × 10³ P388D1.D2 cells in 16-mm wells and then inoculating them the following day with 6 × 10⁶ yeasts in 1 mL of HMM-M medium [16] completed as for F-12 above. The next day, they were washed three times in PBS before in situ fixation in 2% glutaraldehyde at 4°C. After postfixing with osmium tetroxide and dehydrating as above, embedding medium was poured into the wells and the samples were processed in a manner identical to the yeast pellets. In some experiments, yeasts were glutaraldehyde-fixed as described above, washed twice in modified Tyrode’s buffer, and stored overnight in HMM [4] at 4°C prior to addition to macrophages at 37°C in completed HMM-M. Glutaraldehyde-treated fungi were unable to form colonies on solid HMM.

To examine yeasts inside HTE cells, we plated 5 × 10⁴ cells/16-mm well and infected them the following day with 1–5 × 10⁷ yeasts in 1 mL of completed F-12 medium. After 20–24 h, the wells were washed 10 times with completed medium, twice with PBS, and then fixed with glutaraldehyde, as described earlier for macrophages. Samples were treated as above to detect α-(1,3)-glucan by electron microscopy. Approximately 50 yeast cells were examined to evaluate the cell wall architecture.

**Fluorescence microscopy.** For comparative purposes, companion monolayers on coverslips were infected and stained as previously described [4] with Fungigual (provided by J.R. Little, Washington University School of Medicine, St. Louis) to detect chitin and monitor for the presence of allomorphic yeasts [5].

**Results**

**Yeasts in suspension.** Knowing that parental and variant yeasts differ in their cell wall compositions, we looked first at the ultrastructure of broth-grown yeasts. Ruthenium red staining revealed an outermost carbohydrate layer (figure 1A) in the cell wall of all yeasts; organisms from different strains and their variants were indistinguishable (G186A-R, -S, and -HT; UCLA 531-R and -S). As expected, immunogold staining localized α-(1,3)-glucan to the walls of G186A-R (figure 1B) and UCLA 531-R. In the absence of ruthenium red, the cell wall was electron-lucent. With regards to survival in macrophages, ability to kill these phagocytes, and tendency to form allo-morphs, previous work indicates that all chemotype II strains behave identically inside cells [4, 5]. Consequently, the two sets of strains were used interchangeably in the remainder of this study.

**Yeasts infecting macrophages.** Inside P388D1.D2 cells, the parental yeasts retained both the thick, electron-lucent carbohydrates of the cell wall and wall-associated α-(1,3)-glucan (figure 1C). For variants, the thick carbohydrate layer visible in broth-grown organisms was no longer apparent inside these cells. Instead, the cytoplasmic membrane of the yeasts was surrounded by a thin, convoluted layer that was presumed to be the cell wall (figure 1D). As expected, no α-(1,3)-glucan was detected in these walls (not shown). Electron microscopy revealed similar shapes for parental and variant yeasts despite the fact that fluorescence microscopy shows that variants are allomorphic [5]. This apparent paradox is probably a result of inherent limitations of the two methodologies: Entire yeasts are seen by light microscopy, whereas only ultrathin cross-sections of yeasts are examined via electron microscopy.

To determine whether variants actively altered their walls inside macrophages, we used glutaraldehyde pretreated yeasts as an inoculum. Glutaraldehyde treatment prevented allomorphic formation as assessed by fluorescence microscopy. However, this treatment did not prevent changes in the ultrastructure of the walls of variants within macrophages: They still acquired a thin, wavy appearance (not shown).

**Yeasts infecting HTE cells.** Variant yeasts do not form allomorphs in HTE cells [5] but nonetheless had thin, wavy walls inside them (figure 2A). Unexpectedly, the parental strains were found in two different states within these cells. About half the yeasts had thick, electron-lucent walls (figure 2B) but often seemed irregular in shape or had profoundly disarrayed cytoplasm, as though the fungi were dead or dying. Organisms cohabiting a single phagosome were sometimes at different stages of deterioration. The other half of the population had thin, sinuous walls and an intact cytoplasm and were ultrastructurally indistinguishable from variants inside HTE cells (figure 2C). In additional experiments (data not shown), we determined that the thick-walled organisms possessed α-(1,3)-glucan, while the thin-walled fungi did not.

**Discussion**

Parent and variant (S or HTE) strains appear to have similar cell wall ultrastructure when the yeasts are cultured in broth. However, this ultrastructure becomes drastically altered when *H. capsulatum* variants are grown in either macrophages or epithelial cells. The same alterations are seen in variants pretreated with glutaraldehyde, indicating that no active response is required from the yeasts. Instead, the variant cell wall may be particularly sensitive to some enzymatic or other activity within the phagolysosome. Alternatively, the nonprotein portion of the wall may have a passive physicochemical response to the microenvironment within the host cells.
Fluorescence microscopy of macrophages infected with variants reveals a variety of allomorphic shapes, including simple enlarged spheres, barbells, gourds, and kidney beans [5], which are similar to forms found in the tissues of infected mammals and patients. This variety of shapes may relate to the thin, sinuous cell wall seen ultrastructurally. For example, if the thinned walls reflect less rigidity, perhaps osmotic stress could explain allomorphism. As might be expected in such a scenario, enlarged spherical forms predominate. The additional shapes observed might result from uneven stress, perhaps due to local variations in the rigidity of the cell wall with consequent herniation at weaker sites. Consistent with this idea, allomorphs do not form inside cells if the walls of the variants are protected from potential osmotic effects as a result of cross-linking with glutaraldehyde.

The factor(s) that cause allomorphs to form inside macrophages seems to be absent from HTE cells. However, within HTE cells, profound changes can occur in the phagocytized parental strain yeasts. Some of these yeasts appear to undergo degradation within the first day after inoculation. Those that do not have acquired thin, wavy cell walls that are indistinguishable from those of the variants. Thus, the parental phenotype seems unfavorable for survival in HTE cells.

The differences between the microenvironment facing *H. capsulatum* within macrophages and HTE cells are unknown, yet probably influence the yeast morphology and cell wall architecture. In both cases, the fungi are enclosed in membrane-bound vesicles. These have been identified as phagolysosomes of neutral pH in macrophages [17] but have yet to be characterized in HTE cells. Levels of other ions may also differ within the hosting vesicles of these cells, as may the transport of nutrients from the medium or host cell cytoplasm. Both the results reported here and earlier studies [4] indicate that by 24 h after infecting the epithelial cells, about half of the parental...
Figure 2. Ultrastructure of H. capsulatum yeasts inside HTE cells. A. One day after inoculation, thin, sinuous walls (arrow) were again seen on variants (UCLA 531S is shown). B. At same time point in samples inoculated with parental yeasts, electron-lucent wall (arrow) was present on some yeasts (representative strain, UCLA 531R). Cytoplasm of a few remained intact but in many it was in great disarray (*). As seen here, both degrading and apparently intact yeast can occupy same host vesicle. C. Remainder of ingested yeasts (UCLA 531R) had lost thick, electron-lucent wall and acquired wavy, thin one (arrow). Arrowhead: phagosomal membrane. Bar = 500 nm.

yeasts lose α-(1,3)-glucan. The earlier study suggested that HTE cells may actually trigger such a change rather than just selecting for a few α-(1,3)-glucan–negative variants already present within the inoculum. Here it appears that the parental yeasts will be degraded if they do not convert to the thin-walled form quickly enough. Consequently, yeasts harvested from infected epithelial cells after several days uniformly have the variant HTE phenotype [4]. Such yeasts can establish persistent infections of macrophages in vitro [4], and the allomorphic shapes they assume are analogous to those seen in persistently infected patients and animals [6–9]. Our in vitro results suggest that distinctive microenvironments within particular host cells can influence fungal phenotype and the potential for long-term persistence in histoplasmosis.

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References

Atovaquone and Proguanil for the Treatment of Malaria in Brazil

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The purpose of this study was to compare an experimental regimen of atovaquone plus proguanil with the standard regimen of quinine plus tetracycline for the treatment of uncomplicated falciparum malaria. The study was designed as an open, randomized study of men presenting with symptoms of uncomplicated malaria and thick-smear slide confirmation of parasitemia (1000–100,000 ring forms/μL). Subjects were hospitalized for 28 days to insure medication compliance and to rule out the possibility of reinfections. With 77 patients in each group, the cure rates were 98.7% and 100% for atovaquone plus proguanil and quinine plus tetracycline, respectively. The parasite clearance times (mean, 56 h) and fever clearance times (mean, 19 h) were significantly shorter in the atovaquone plus proguanil group, and there were significantly fewer side effects in the atovaquone plus proguanil group. Atovaquone plus proguanil is an efficacious, easily administered, safe regimen for the treatment of uncomplicated, multidrug-resistant falciparum malaria in Brazil.

Multidrug-resistant falciparum malaria continues to be a major public health problem in developing countries, requiring new treatment regimens nearly every decade. The problem is especially severe in Brazil, which has experienced a 3-fold rise in malaria during the past decade, to nearly 600,000 infections annually, approximately half of which are falciparum [1]. Nearly all of the malaria is confined to the Amazon region, in which migrant populations, great distances, and poor access to diagnosis and treatment are major obstacles to malaria control. Presumptive treatment without diagnosis has led to overuse of antimalarials and is probably a significant factor in the generation of parasite resistance. Migrant populations can rapidly disseminate resistant strains throughout the region. During the past decades, parasite drug resistance to chloroquine, pyrimethamine-sulfadoxine, and to some extent, quinine [2] has developed at an alarming rate. Currently, the recommended regimens for uncomplicated falciparum malaria in order of priority are quinine plus tetracycline for 7 days, quinine alone for 10 days, and a single dose of mefloquine [3]. The first two regimens have problems with poor compliance and many side effects. Half the patients do not comply for >3 days (de Alencar FEC, unpublished data). Mefloquine is expensive, and because of its long half-life, resistance may develop rapidly through reinfections in malaria-endemic areas.

As in other areas of the world faced with multidrug resistance, the options for alternatives are few and problematic. Increasing the dose of existing regimens may lead to toxicity and only delay the development of resistance. Combining different regimens raises the cost and the chance of side effects and leads to poor compliance. The development of new drugs is costly and slow (relative to the development of parasite resistance), and there is always the possibility of cross-resistance to existing drugs with similar chemical structures. New drugs, widely used in Southeast Asia, are the artemisinin compounds. These are also available in Brazil, where they are...