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Phenotypic Variation and Persistence of *Histoplasma capsulatum* Yeasts in Host Cells

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Many *Histoplasma capsulatum* strains spontaneously give rise to variants during broth culture or subsequent to ingestion by epithelial cells. Unlike their parents, these variants are defective in killing macrophages and lack a major cell wall constituent, α-(1,3)-glucan. Inside macrophages, where the variants can persist for several weeks, they adopted an unusual morphology strikingly similar to that reported in the tissues of persistently infected humans or animals. These yeasts were often enlarged or misshapen (allomorphic), but were viable. Decreased cytotoxicity for macrophages was more strongly associated with allomorph formation than was the absence of cell wall α-(1,3)-glucan. Allomorphs were also formed in rat and mouse resident macrophages, but not in hamster trachea epithelial cells, indicating that host cell type influences the morphology of these yeasts. We propose that during *H. capsulatum* infection of mammalian hosts, spontaneous variants arise which can be recognized by their unusual morphologies. In contrast with their virulent parents, such variants "peacefully coexist" within macrophages, potentially contributing to the establishment of latency in vivo.

Evolutionary selection often favors organisms which can survive without destroying their ecological niche. In the case of intracellular pathogens, this pressure frequently results in chronic or latent infections. *Histoplasma capsulatum* is a fungal pathogen adept at causing persistent intracellular infections of mammals. Months to years after either inapparent or overt disease, an imbalance in immunity and/or other undefined host factor(s) can result in reactivation of the initial infection (for reviews, see references 8 and 9). Little is known regarding how *H. capsulatum* establishes its latent state.

H. capsulatum chemotype II strains, defined as having α-(1,3)-glucan in their cell walls (20), form rough colonies on solid medium and rapidly destroy P388D1.D2 macrophage-like cells. When tested, destruction of these cells has generally correlated well with lethality for mice. These strains also spontaneously spawn variants which are defective in killing both mice and P388D1.D2 cells (14).

We have previously described two types of variants. One can be enriched from broth culture by repeated passaging of yeasts that grow in a dispersed, slowly sedimenting form in contrast to their clumping parental strains. Variants isolated in this fashion have a smooth colony phenotype (hence, the suffix "S" is added to the strain name) and lack α-(1,3)-glucan (14). Passaging of parental strains through cultured hamster trachea epithelial (HTE) cells selects for a second type of variant (referred to as "-HTE variants") with a similar smooth colony phenotype and also lacking α-(1,3)-glucan. Slight but consistent differences in colony color and texture suggest that the two types represent distinctive variations. Variants can survive within P388D1.D2 cells with little overall damage to the monolayer for at least a 3-month period (11). Here, we further examine persistence by these organisms and relate the unexpected finding that unlike their parents, the variants assume unusual morphologies inside macrophages. The observed shapes are reminiscent of organisms found in nonpulmonary tissues of patients or animals with chronic or persistent infections (8).

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MATERIALS AND METHODS

Yeast strains. *H. capsulatum* strains G184A, G186A, G217B, and *H. capsulatum* var. *duboisii* RV26821 were obtained from the American Type Culture Collection. UCLA 531 was acquired from Dexter Howard (University of California School of Medicine, Los Angeles). George Kobayashi (Washington University School of Medicine, St. Louis, Mo.) supplied the Downs strain, which we found to contain α-(1,3)-glucan in its cell wall. This strain has been shown to have reduced virulence in mice (15, 17), but is lethal for P388D1.D2 macrophages according to a previously described assay (11), suggesting that its virulence defect is unrelated to killing of macrophages. In earlier work (11, 14) we described the derivation of -S and -HTE variants and tabulated their characteristics along with those of most of the other strains used in this study (11). We have since derived Downs-S which, as expected, is defective in killing macrophages.

In keeping with our previous nomenclature, we use the suffix "R" to denote all macrophage-virulent, rough colony, α-(1,3)-glucan-containing parent strains. The methods for culturing the yeasts and preparing them for inoculation onto monolayers have been reported (11). The viability of yeasts was assessed as previously described (2). Under routine culture conditions, greater than 90% of our inoculum was viable.

Culture of mammalian cells. P388D1.D2 is a randomly selected clone from P388D1, whose culture conditions have been described elsewhere (10). The isolation and characteristics of HTE cells, a nontransformed epithelial cell line derived from hamster trachea, have also been described previously (12). For all experiments, we seeded 2×10^4 P388D1.D2 cells or 1×10^4 HTE cells into 16-mm-diameter wells containing 12-mm-diameter glass coverslips. Cells were incubated in a humidified 95% air–5% CO_2 incubator at 37°C.

We harvested macrophages from the peritoneal cavities (3) of male Sprague-Dawley rats (Sasco; Omaha, Neb.) or HSD:NSA(CF1)Br female mice (Harlan Sprague-Dawley, Inc., Indianapolis, Ind.) by using cold minimal essential medium (GIBCO) without supplements. These cells were plated at 5×10^5 per well in minimal essential medium containing 9 μg of gentamicin per ml and 1% fetal bovine serum (HyClone), which had been heat inactivated for 30 min at 56°C. After 2 h, we washed the coverslips six times in phosphate-buffered saline and replaced the medium. This was repeated 2 h later, but this time the monolayers were reincubated overnight in medium containing 10% heat-inactivated fetal bovine serum. After one more wash the next day, the macrophages were returned to the incubator in the latter medium for a final 24 h before the experiment. This procedure minimized the number of nonadherent cells.

Infection of cultured cells. Unless otherwise specified, we inoculated P388D1.D2 cell monolayers with a yeast-to-host cell ratio of 1:5 as previously described (11). Because of the greater sensitivity of rat and mouse macrophages to *Histoplasma* spp., we used only a 1:50 ratio for these cells. HTE cells received a multiplicity of infection of 2 yeast cells per cell. Infected monolayers were stained with Fungiqual (11) at appropriate times. For indicated experiments, yeasts were treated with antiserum against *H. capsulatum* (Immuno-Mycologics, Norman, Okla.) for 1 h at 37°C and washed twice before being diluted for use as

an inoculum. A minimum of 200 yeast cells were surveyed to determine the proportion which were allomorphic (barbell, gourd, or kidney bean shaped; elongated oblongs; or enlarged spheres) rather than the prototypical teardrop shape. With these criteria, it was possible to determine blindly which samples were inoculated with variants and which were inoculated with parental strains.

Loading lysosomes with fluoresceinated dextran. To monitor the viability of ingested yeasts, we preincubated P388D1.D2 cells for 4 h with 10 mg of fluorescein-isothiocyanate-labeled dextran (FITC-dextran; Paul Schlesinger, Washington University School of Medicine) per ml of completed F-12 medium as previously described (10). Monolayers were inoculated the next day with one yeast cell per five macrophages. At various times thereafter, we examined internalized organisms by fluorescence microscopy.

Fluorescent antibody staining for α-(1,3)-glucan and *H. capsulatum* antigens. At indicated time points, infected monolayers were fixed for 5 min with methanol and examined by indirect double immunofluorescence staining as previously described (11). Either a standard epifluorescence microscope (Leitz, Rockleigh, N.J.) or a confocal microscope (MRC500; Bio-Rad, Hercules, Calif.) was employed to evaluate samples. We used a minimum of three separate samples, examining at least 200 yeast cells in each, to determine the percentage of organisms possessing α-(1,3)-glucan in their cell wall.

RESULTS

Viability and morphology of intracellular variant yeasts. We were unable to find a commercial staining technique which could distinguish between live and dead yeasts inside of macrophages. Consequently, we investigated an earlier observation that suggested differential staining of live and dead organisms inside phagolysosomes prelabeled with FITC-dextran (unpublished data). In those experiments, it appeared that FITC-dextran permeated dead yeasts, but only outlined live ones. Others have similarly observed that dead mammalian cells are permeable to fluoresceinated proteins, while live cells are impermeable (7).

To confirm that FITC-dextran could distinguish between live and dead intracellular yeasts, we inoculated prelabeled P388D1.D2 cells with a culture of 99% viable G186AS yeasts, with uniformly dead yeasts (boiled or methanol treated), or with a population of organisms which was 29% viable (after a 10-min treatment with 10% formalin). Subsequently we compared these values with those for the fraction of internalized organisms encircled by FITC-dextran. Three hours postinoculation, 99% of the variants from the live inoculum were outlined with fluorescence. In contrast, all boiled or methanol-treated variants were solidly fluorescent. The proportion of formalin-treated yeasts rimmed with FITC-dextran (21%) was similar to the portion viable in the original inoculum, confirming that the proportion of live yeasts within macrophages could be estimated from the number of organisms outlined by fluorescence. Furthermore, 6 days later, 98% of the variants remained rimmed with FITC-dextran.

The number of variant yeasts found inside the cells appeared to increase with time. After infecting macrophages with parental or variant yeasts (1 yeast cell per 10 host cells), we stained the monolayers with Fungiqual to detect cell wall chitin, and thus visualize the yeasts. At 1 day postinoculation, there was no apparent difference in the colonization of P388D1.D2 cells by the two strains: a low proportion of the macrophages were infected, and these contained only a few organisms. By 6 days, numerous variants were found in nearly all of the macrophages in the monolayer. Parental strains had destroyed the monolayer by this time, while heat-killed organisms were very difficult to find. Because of difficulties in washing away extracellular yeasts without also washing away or damaging infected macrophages, we were unable to determine whether the allomorphs themselves were multiplying or whether infected cells occasionally lysed and the released yeasts multiplied before reinfecting new macrophages. Regardless, monolayers remain persistently infected for up to 3 months without being destroyed (11).

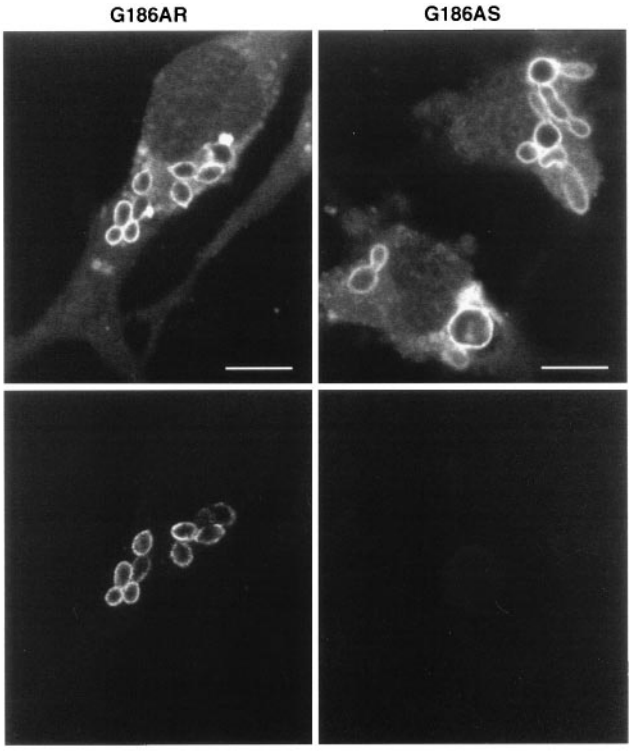

FIG. 1. Aberrant morphology of variant *H. capsulatum* yeasts in macrophages. Monolayers were inoculated 1 day previously, fixed, and stained for double immunofluorescence examination by a confocal microscope. Infected cells were examined for *H. capsulatum* antigens (top row) and monitored for α-(1,3)-glucan (bottom row). Staining with Fungiqual to detect chitin provided images identical to those in the top row. The teardrop shape of G186AR yeasts is typical of all parental strains. In contrast, most ingested G186AS yeasts and other variants were aberrantly shaped and stained only with antiserum to *H. capsulatum*. Bars, 10 μm.

Inside macrophages, the vast majority of G186AR yeasts have a teardrop shape, as do all strains in liquid culture. In contrast, internalized G186AS yeasts were often spherical and bloated, some having short mycelial extensions (Fig. 1). Other yeasts were more barbell-like, gourd shaped, or had one bloated form separated from one or two normally shaped organisms by septae. Similar forms can also be seen, although at a low frequency (≤5%) during broth culture of every strain we possess. We adopted the term "allomorph" (Gr. n. *allo*, other; *morph*, shape) to refer to these oddly shaped, yet viable yeasts.

Factors that influence allomorph formation. We subsequently examined the shapes of yeasts inside P388D1.D2 cells infected with other strains and their variants. The strains examined included chemotype I organisms (e.g., G217B), which lack cell wall α-(1,3)-glucan but still kill both macrophages and mice, and chemotype II strains, whose parental strains (-R) all possess this cell wall polymer (G186A, Downs, UCLA 531, and *H. capsulatum* var. *duboisii* RV2682). All -S and -HTE chemotype II variants assumed an allomorphic appearance inside macrophages. However, strains which kill macrophages and mice (G217B and the R strains) formed no more allomorphs in P388D1.D2 cells than they do in broth culture. The percentages of yeasts which were allomorphic in macrophages clearly increased with time for -S and -HTE variants, rising to 89% ± 5.0% within 1 day of inoculation. No such increase occurred for parental strains (Fig. 2). Just as in P388D1.D2, variants became allomorphic in cultured mouse and rat resident peri-

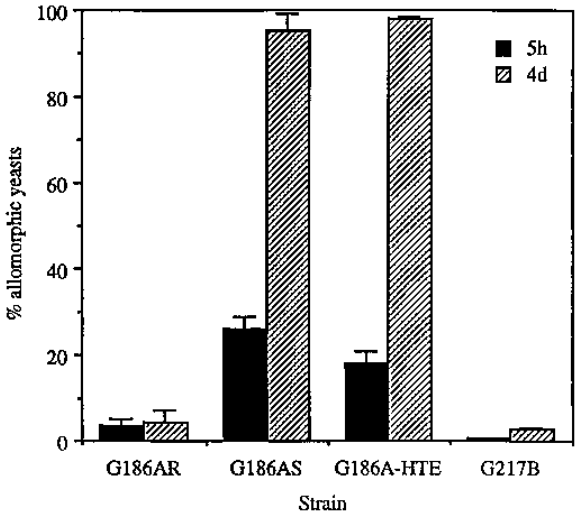

FIG. 2. Effect of time on the percentage of yeasts which are allomorphic in various strains. P388D1.D2 cells were examined by staining with Fungiqual at 5 h and 4 days after inoculation with various strains of *H. capsulatum.*

toneal macrophages (data not shown). In contrast, no strain formed allomorphs inside HTE cells.

Since very large inocula of variants can kill macrophages (unpublished observation), we investigated the possibility that a large multiplicity might also overcome whatever macrophage factors lead to allomorph formation. Instead, allomorphs continued to be formed, even at multiplicities of infection sufficiently high to destroy P388D1.D2 cells in a few days (five yeast cells per host cell). Antiserum specific for *H. capsulatum* also failed to inhibit allomorph formation. However, as expected, variants killed with heat or glutaraldehyde did not form allomorphs.

The phenomenon of reactivation histoplasmosis implies that a latent form of *Histoplasma* cells may have the capacity to become more cytotoxic under the appropriate conditions. Because destruction of macrophages is associated with the presence of α-(1,3)-glucan in the cell walls of chemotype II strains, we examined whether any of the intracellular allomorphs possessed this polymer.

In contrast to broth-grown G186AS yeasts [which are completely lacking in α-(1,3)-glucan], a small proportion (3.1% ± 1%; $n = 3$) of G186AS yeasts inside P388D1.D2 macrophages regained this polymer in their cell walls as early as 1 day postinoculation, despite an allomorphic shape (Fig. 3). In some cases, confocal microscopy revealed that only a portion of an individual yeast had α-(1,3)-glucan. There was no obvious change in the proportion of organisms possessing this polysaccharide over time (day 2, 4.0% ± 0.3%; day 3, 3.4% ± 0.3% [$n = 3$]). In most cases, yeasts within a single macrophage shared the same α-(1,3)-glucan phenotype.

DISCUSSION

While *H. capsulatum* -S and -HTE variants are defective in killing macrophages, the intracellular variants remain viable for at least 6 days postinoculation. During this time, the variants appear to increase in number, and at least some of them persist for up to 3 months (11). However, inside macrophages, the variants undergo a drastic change in morphology which begins to be evident as early as a few hours after infection: the usual oval or teardrop organisms become allomorphic, often being enlarged and spherical or otherwise misshapen. This unusual change in shape was evident in variants of strains from four separate evolutionary classes (26). Just as *Histoplasma*-specific antiserum treatment reportedly has no effect on the viability of the organism in macrophages (13), antiserum also fails to inhibit allomorph formation.

To date, all variants we possess form allomorphs in macrophages and lack α-(1,3)-glucan when grown in broth culture. However, several factors indicate that a defect in killing macrophages is better correlated with allomorph formation than the absence of α-(1,3)-glucan. First, there are at least a few viable allomorphs in broth cultures of all parental strains, regardless of cell wall composition. These organisms do not differ from the remainder of the population in terms of their α-(1,3)-glucan content. Second, whereas chemotype I strains (e.g., G217B) lack this polysaccharide, they do kill macrophages and overwhelmingly retain a normal shape inside the phagocytes. Finally, occasional variants regained α-(1,3)-glucan synthesis inside macrophages, but remained allomorphic.

Allomorphs bear a striking resemblance to Darling's first description of histoplasmosis in 1906 (4). The unusual morphological forms that he encountered resulted in his erroneous classification of the etiological agent; these forms were carefully depicted in drawings purporting to show a "protozoan" growing inside cells. Subsequently, there were numerous accounts of abnormally large (up to 20 μm) or irregularly shaped yeasts in patients, animals, or tissue explants (for a review, see

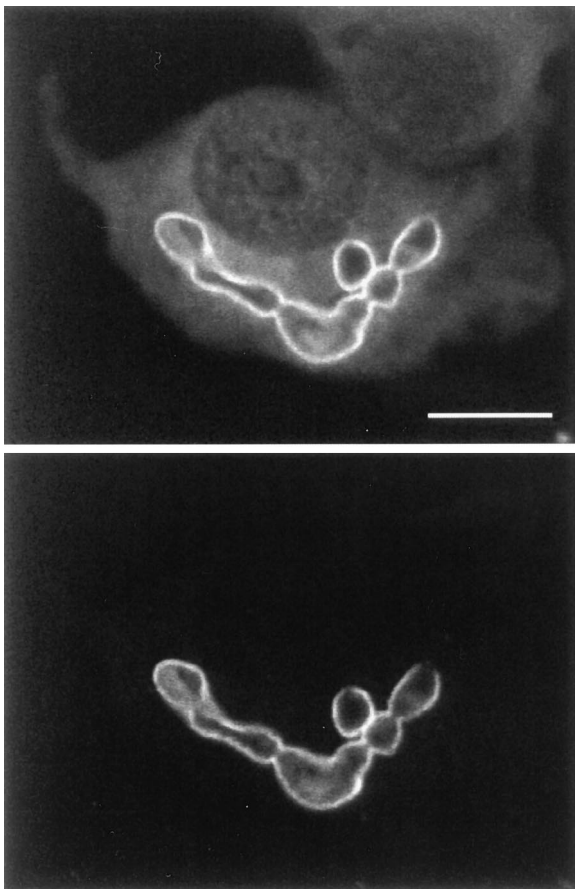

FIG. 3. Occasional variants regain α-(1,3)-glucan inside P388D1.D2 macrophages. Samples were prepared as described for Fig. 1, with the top panel showing immunofluorescence staining of *H. capsulatum* antigens and the bottom panel showing detection of α-(1,3)-glucan. Bar, 10 μm.

reference 8). The difficulty in staining these yeasts by conventional means led to the now common silver staining of suspect tissues. In this manner, buds or abortive hyphae were revealed on some enlarged forms, as can also be seen in cell culture. Necrotic tissues sometimes contained extracellular allomorphs (for a review, see reference 18), supposedly released by degenerating cells.

In the 1950s (18), there was much discussion of whether the "giant" forms were an alternate growth state of *H. capsulatum* or a second fungus (e.g., *Blastomyces* spp.) Two observations led to the conclusion that infection with *H. capsulatum* alone could be manifested by yeasts which vary greatly in morphology. First, *H. capsulatum* cultures from patients whose tissues had large yeasts were symptomatically distinct from patients with blastomycosis (1, 27). Second, large forms could be demonstrated upon experimental infections of animals with pure cultures of *H. capsulatum* (5, 22, 23). On the basis of our in vitro studies, we propose that variant yeasts may be at least partly responsible for the diversity in shape.

Enlarged yeasts have been most consistently reported in African histoplasmosis, which is caused by *H. capsulatum* var. *duboisii*. This variety is considered relatively avirulent for animals (16, 23), is often associated with nonpulmonary infections, and has a tendency to become persistent (for a review, see reference 21). Moore (18) felt that the variety *duboisii* (at that time considered to be a species) was in fact the tissue form of *H. capsulatum*. Intriguingly, even with non-*duboisii* strains, allomorphic yeasts are frequently reported in nonpulmonary tissues to which the organism might have migrated during either overt or inapparent infections (24, 25). Some of these tissues (e.g., skin, heart, brain, adrenal glands, oropharyngeal cavity, and gastrointestinal tract) have relatively few macrophages. Others, such as the liver, have numerous mononuclear phagocytes. Large yeasts from fresh biopsies of various organs form normal-sized yeasts in culture (6). Subsequent injection of these in vitro-passaged yeasts caused no apparent illness in animals. Nonetheless, autopsies 6 to 8 months later revealed the presence of chronic lesions. Together, these observations suggest that, as a prelude to or as a result of dissemination, yeasts likely come into intimate contact with and possibly infect cells other than alveolar macrophages. Such interactions may encourage development of variants prone to establish persistent infections which are characterized by allomorphic yeasts.

O'Hern (19) found that resistance to histoplasmosis in hamsters correlated with the presence of large yeasts within the liver. Survivors had enlarged yeasts in this organ, whereas those who died did not. It was subsequently demonstrated that after the large yeasts become evident, explants from several organs of resistant animals can inhibit the multiplication of the yeasts in vitro. While allomorphs continued to reproduce in cell culture over the time examined here, such multiplication may eventually become limited. Likewise, intact tissues might possess additional factors which restrict reproduction. The appearance of enlarged organisms in animals seems to reflect a point of balance between host and pathogen at which neither is apt to induce significant damage in the other. The existence of such a balance point is consistent with our previous designation of the variants as avirulent: they tend to kill neither mice nor macrophages despite establishing inapparent persistent infections in both. In contrast, parental strains not only survive within macrophages but also kill both these phagocytes and mice.

While inapparent persistence of variants may generally occur, a natural question is whether variants can regain cytotoxicity for macrophages and thus be responsible for reactivating histoplasmosis. High multiplicities of variants can kill macrophages, so it is conceivable that an alteration in the host (e.g., immunosuppression) may favor variant multiplication or macrophage deterioration and likewise favor reactivation. As additionally reported here, a low, but apparently stable, proportion of variants can at a minimum regain the ability to synthesize α-(1,3)-glucan. The fact that yeasts within a single macrophage tend to have the same α-(1,3)-glucan phenotype suggests that they are daughters of a single yeast which regained α-(1,3)-glucan after infecting the phagocyte or that some property of individual macrophages promotes α-(1,3)-glucan synthesis. This trait is associated with virulence in chemotype II strains and may be one of many phenotypes which directly or indirectly contribute to virulence. We are presently attempting to isolate α-(1,3)-glucan-positive organisms from infected macrophages to determine if cytotoxic traits are regained as well.

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