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Cell Walls from Avirulent Variants of *Histoplasma capsulatum* Lack α-(1,3)-Glucan

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Cell wall composition of isogenic virulent-avirulent strain pairs of *Histoplasma capsulatum* varied markedly with respect to α-(1,3)-glucan content. When yeast cell walls were fractionated by standard techniques, the avirulent strains contained up to 1,000-fold less α-(1,3)-glucan than did their virulent parents. No α-(1,3)-glucan could be detected on the surface of the avirulent strain yeast cells if we used a mouse monoclonal antibody that recognized this polymer. A similar relationship between virulence and α-(1,3)-glucan has been described for *Paracoccidioides brasiliensis*. α-(1,3)-Glucan is also found in several other pathogenic fungi and may thus be an important common virulence determinant.

Histoplasma capsulatum is a dimorphic fungal pathogen that is able to survive and grow inside macrophages. One of the initial defense mechanisms available to macrophages, the release of toxic oxygen metabolites, is not triggered by phagocytosis of *H. capsulatum* (6). However, the phagocytosed yeast cells are exposed to degradative enzymes and other fungicidal factors when lysosomes fuse with phagosomes (7). Like *Mycobacterium lepraemurium* (2, 8), *Salmonella typhimurium* (3), and *Aspergillus fumigatus* conidia (13), virulent strains of *H. capsulatum* are able to proliferate within the harsh environment of the phagolysosome.

How these organisms survive the onslaught of degradative enzymes is not known, but one way could be through protection by a cell wall layer resistant to the lysosomal contents. Previously, we described the selection of spontaneous avirulent mutants from standard virulent strains of *H. capsulatum* (12). Strain G-186AR is a clone from the American Type Culture Collection virulent strain G-186A (ATCC 26029). Strain G-186AS is a cloned avirulent variant selected from G-186AR (12). These strains and similar virulent-avirulent pairs were shown to have comparable in vitro growth rates as well as virtually identical cell-associated protein profiles. In this paper, we examine nonprotein components from the yeast cell walls of several isogenic virulent and avirulent strains of *H. capsulatum*.

H. capsulatum yeast cells grown for 72 to 96 h at 37°C in the defined medium HMM (21) were collected by centrifugation at 800 × *g* and suspended in phosphate-buffered saline with approximately one-third volume of acid-washed glass beads (0.45 μm; Fisher Scientific Co., Pittsburgh, Pa.) added. The cells were then disrupted by vortexing vigorously for 4 min. Cell walls were collected by centrifugation at 500 × *g* for 5 min and then washed with 1% sodium dodecyl sulfate followed by repeated (5 to 10) washes with distilled water.

The cell wall polysaccharides were then fractionated according to their differing solubilities in alkali (15). *H. capsulatum* cell walls processed in this way contain chitin and β-glucans in fraction 1, α-(1,3)-glucan in fraction 2, and β-glucans in fraction 3 (11, 15). Each cell wall fraction was hydrolyzed in vacuo with 4 N HCl at 110°C for 6 h. After acid hydrolysis, the sugars were separated in formic acid-acetic acid-water (1:4:50, pH 1.9) with high-voltage paper electrophoresis (80 V/cm, 30 min) and identified by comparison with standards. The avirulent strain G-186AS had almost no sugar present in fraction 2 [α-(1,3)-glucan] (Fig. 1). Quantitation of the glucose content in fraction 2 was achieved with the phenol-sulphuric acid assay (5). With this technique, α-(1,3)-glucan was up to 1,000-fold less abundant in the avirulent strain G-186AS than in its virulent counterpart (Table 1). A similar decrease in the amount of hexose in fraction 2 was observed for avirulent clones selected from strain G-184AR (data not shown).

These results were supported by experiments with a mouse monoclonal antibody, MOPC 104E (Sigma Chemical Co., St. Louis, Mo.), which recognizes α-(1,3)-glucan. Log-

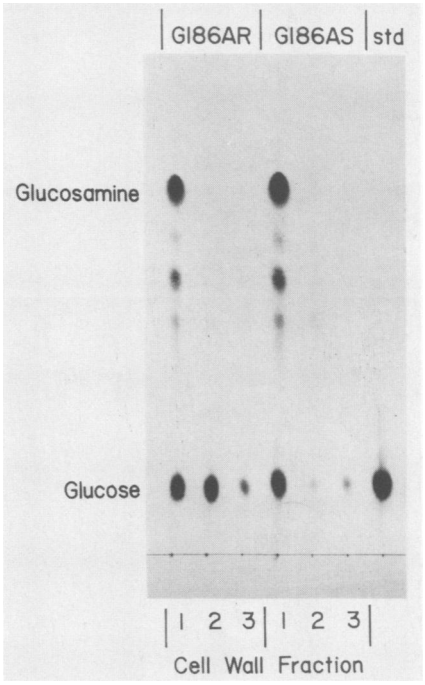

FIG. 1. High-voltage paper electrophoresis of sugars from acid-hydrolyzed cell wall fractions of *H. capsulatum* yeast cells. Fraction 1 contains chitin (a polymer of *N*-acetylglucosamine) and β-glucans, fraction 2 contains α-(1,3)-glucan, and fraction 3 contains β-glucans. The right lane contains 5 μg of glucose as a standard.

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TABLE 1. Quantitation of α-(1,3)-glucan in *H. capsulatum* yeast cell walls: strain differences and culture conditions

Medium[a]	Strain	Glucose (μg/10^8 cells) in α-(1,3)-glucan fraction[b]
BHI	G186AR	108.3
	G186AS	<0.1
BHI + 7.5% FBS	G186AR	82.9
	G186AS	<0.1
HMM	G186AR	104.4
	G186AS	<0.1

[a] BHI, Brain heart infusion broth; FBS, fetal bovine serum; HMM, a defined medium for *H. capsulatum* (21).

[b] As determined by phenol-sulfuric acid assay of hexose in cell wall fraction 2 (see text); data representative of three experiments.

phase yeast cells were collected by centrifugation and washed with 1% bovine serum albumin in phosphate-buffered saline (used for all wash steps). Yeast cells were resuspended in bovine serum albumin–phosphate-buffered saline, and antibody was added to a concentration of 100 μg/ml in a total volume of 250 μl. After incubation at room temperature overnight, the yeast cells were then washed twice and resuspended in 200 μl of bovine serum albumin–phosphate-buffered saline. A fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin M (Sigma) was added to the resuspended yeast cells (final dilution, 1:20) and allowed to react for 2 to 4 h at room temperature. The yeast cells were again washed twice and then examined with a Leitz fluorescence microscope. All cells of the virulent strains G-186AR and G-184AR fluoresced brightly, while the corresponding avirulent strains G-186AS and G-184AS showed no reaction with this anti-α-(1,3)-glucan antibody (Fig. 2). Controls for nonspecific binding with the monoclonal immunoglobulin M against Thy 1.2 (ICN Biochemicals, Naperville, Ill.) were negative for both strains G-186AR and G-186AS (data not shown). Therefore, the MOPC 104E monoclonal antibody was detecting α-(1,3)-glucan rather than fortuitously binding to an Fc-like receptor on the cell surface of the virulent strains.

α-(1,3)-Glucan has been strongly implicated in the virulence of *Paracoccidioides brasiliensis*, another dimorphic fungal pathogen (14, 18). Studies by San-Blas et al. (18) have shown that reduced cell wall α-(1,3)-glucan in nitrosoguanidine-induced mutants or laboratory-passaged strains can be directly correlated with a decrease in virulence for mice. Conversely, *P. brasiliensis* mutants with greater amounts of α-(1,3)-glucan have increased virulence (17). Additional experiments with the avirulent mutants demonstrated that α-(1,3)-glucan production could be restored after culture in medium containing fetal calf serum or after passage through hamsters (16, 19). When cultured in these ways, the mutants also regained their virulent phenotype.

In contrast, our avirulent variants of *H. capsulatum* did not synthesize α-(1,3)-glucan when cultured in fetal bovine

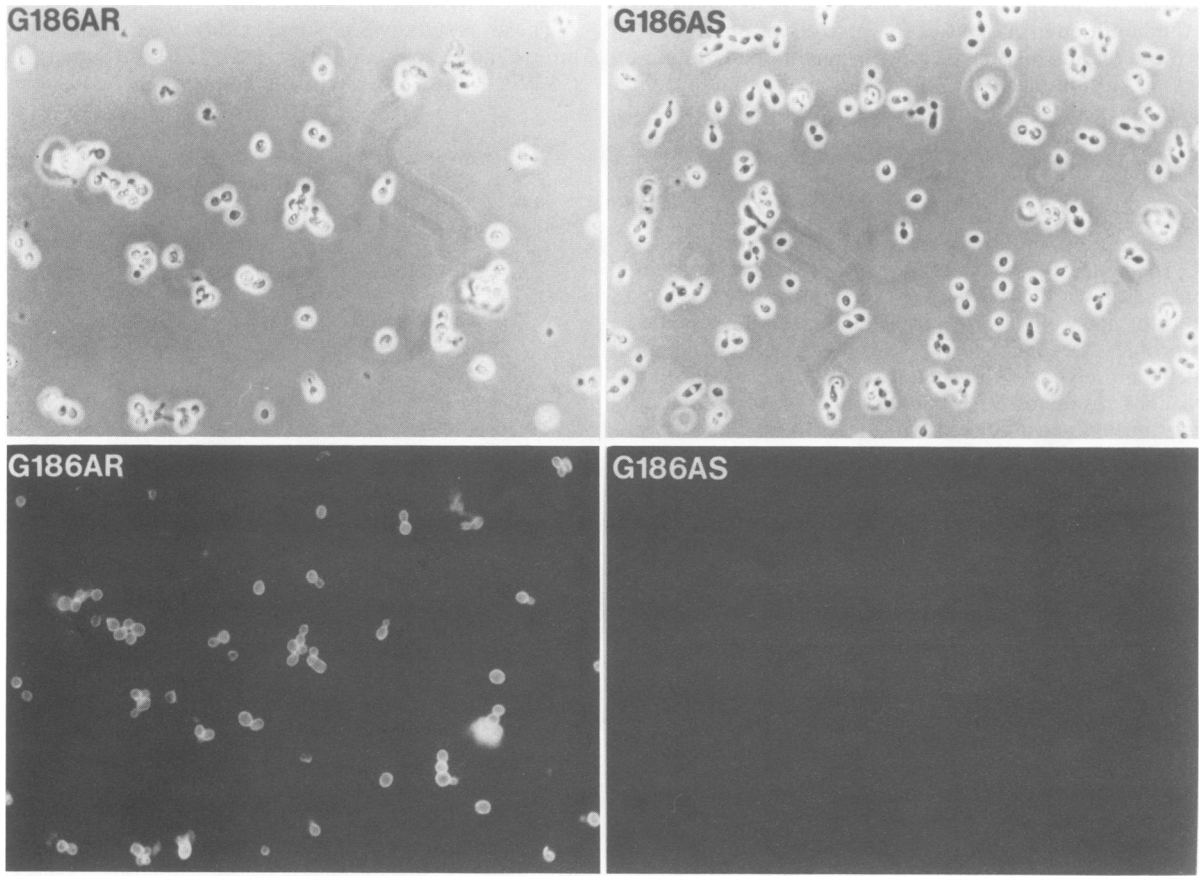

FIG. 2. Yeast cells of *H. capsulatum* virulent strain G186AR and avirulent variant G186AS, reacted with MOPC 104E [anti-α-(1,3)-glucan] and stained with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin M. Top panels, Phase-contrast microscopy; bottom panels, fluorescence microscopy of the same field. Magnification, ×425.

serum (HyClone, Logan, Utah) (Table 1). Earlier experiments also failed to generate virulent revertants by in vitro or in vivo selection schemes (12). Unlike the case with the *Paracoccidioides* example, the ability of our avirulent variants of *H. capsulatum* to synthesize α-(1,3)-glucan appeared to be irreversibly lost.

Domer (4) has previously classified virulent strains of *H. capsulatum* into two distinct groups based on the α-(1,3)-glucan content of their yeast cell walls. Strains designated chemotype II have large amounts of α-(1,3)-glucan, while those designated chemotype I have barely detectable levels. Among these strains there was no correlation between the level of virulence and the amount of α-(1,3)-glucan. Why certain virulent *H. capsulatum* strains appear to lack this polymer is not clear, but one explanation follows the *Paracoccidioides* example: perhaps the chemotype II strains constitutively produce α-(1,3)-glucan, while the chemotype I strains are able to regulate its production. Thus, it is possible that both chemotypes produce this polysaccharide in vivo but that the chemotype I strains repress synthesis of α-(1,3)-glucan when grown under laboratory conditions. Indeed, the initial chemotype studies by Domer used yeast cells that were maintained in the laboratory by continuous passage. One of the chemotype II strains (G-184A) had lost a considerable amount (39.5%) of its α-(1,3)-glucan during passage over 4 years (15).

Prompted by the *P. brasiliensis* analogy, we have attempted to induce α-(1,3)-glucan production in two chemotype I strains (G-217B and G-222B) as well as in our avirulent strains (G-186AS and G-184AS). The addition of fetal bovine serum or human serum (Sigma) at up to 10% in media at various pHs (5.0 to 8.0) and with different carbon sources (glucose, glycerol, or lactic acid) did not detectably change the levels of cell wall α-(1,3)-glucan in any strain tested. Intracellular growth of strains G-217B and G-222B for up to 8 days in P388D1 macrophagelike cells also did not increase α-(1,3)-glucan production to detectable levels. These experiments (data not shown) suggest that chemotype I strains synthesize α-(1,3)-glucan only in vivo or that it is present in vitro at some low level which is protective but not detectable by our assay systems.

Specifically how α-(1,3)-glucan in the cell wall alters the virulence of certain *H. capsulatum* strains is not understood. Glucans have been shown to affect the normal metabolism of macrophages (20), though no studies have involved this particular polysaccharide. The lack of α-(1,3)-glucan in the avirulent mutants may result in some secondary effect on the cell wall architecture. Proteins may not be incorporated or positioned correctly in the mutant cell wall and/or may have altered function because of improper orientation. In addition, the permeability or secretory ability of the yeast cells may be affected. For example, we have previously shown that small peptides are released in different amounts from the virulent-avirulent strain pairs (12).

It has been demonstrated by others that virulence in *P. brasiliensis* is directly correlated with α-(1,3)-glucan content in its cell wall (14, 17, 18). Other medically important fungi also contain significant amounts of this uncommon glucose polymer in their cell walls (1, 9, 10). We present evidence here that α-(1,3)-glucan represents the major phenotypic difference between certain virulent strains of *H. capsulatum* and their avirulent mutants. The function of this polysaccharide may be structural, providing a matrix for proper presentation of proteins involved in virulence. It may passively regulate the secretion of certain factors which allow the yeast cells to survive within macrophages; it may simply act as a protective layer, preventing host defense mechanisms (such as lysosomal enzymes) from eliminating this pathogen. Although α-(1,3)-glucan is probably not the only virulence determinant of *H. capsulatum*, our paired virulent-avirulent strains should facilitate biochemical and genetic analyses of its regulation and role in virulence.

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