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Channels (Pores) Observed in the Cell Wall of the Pathogenic Yeast *Cryptococcus neoformans* Using High-Pressure Freezing/Freeze Substitution and Immunolabeling

Jeffrey G. Ault,[*] Vishnu Chaturvedi,[**] and Sudha Chaturvedi[**]

[*] EM Core Facility and [**] Laboratory of Mycology, Wadsworth Center, New York State Department of Health, Albany, NY 12203, USA

Cryptococcus neoformans, an encapsulated yeast, causes serious meningoencephalitis in both immunocompromised and normal individuals and therefore is of medical and public health concern. The capsule and wall of *C. neoformans* makes it hard-to-fix. We have developed high-pressure freezing/freeze substitution and immunolabeling protocols to study the cellular structure and processes of this pathogenic fungus, taking into account personnel safety. To alleviate the possibility of contamination and infection of EM personnel, cells grown overnight in yeast extract peptone broth were prefixed with 2% paraformaldehyde/0.2% glutaraldehyde in 0.1 M PBS for 2 hours at room temperature and washed with buffer before loading them into planchets for the Balzers HPM 010 high-pressure freezer (HPF). This rendered the yeast cells harmless. Here, high-pressure freezing was not used to quickly freeze and preserve cellular processes in living cells, but rather to prepare prefixed cells for freeze substitution (FS), where they were slowly dehydrated using 100% methanol at -90° C for 72 hours than at -60° C for 48 hours. The cells were then brought slowly to room temperature, washed twice in 100% methanol and embedded in LR White. Some of the prefixed cells were freeze-substituted in 1% osmium tetroxide in acetone and embedded in Epon/Araldite for ultrastructural comparisons.

Prefixing *C. neoformans* cells before high-pressure freezing had minimal effect on ultrastructure. The ultrastructure of prefixed *C. neoformans* cells that were osmicated during FS and embedded in Epon/Araldite (Fig. 1) was preserved as well as that of *Saccharomyces cerevisiae* cells that were high-pressure frozen live and then processed in a similar way. The immunolabeling protocol preserved cellular ultrastructure reasonably well and revealed good immunolocalization of a mouse monoclonal antibody against the capsular molecule 2H1, denoted by smaller 5-nm gold particles, and of affinity-purified rabbit polyclonal antibodies against Cu, Zn superoxide dismutase (SOD1p), denoted by larger 15-nm gold particles. Immunolabeling showed that both antigens are secreted out of the cell via channels through the cell wall (Fig. 2). We believe that this is the first report where such channels have been observed in any pathogenic fungus. In summary, harmful exposure to pathogenic organisms is prevented and ultrastructure is well-preserved by prefixing cells before high-pressure freezing. Such a precaution can help protect personnel in public health or counter-bioterrorism laboratories where pathogenic organisms are studied ultrastructurally. Here, well-preserved cells were obtained for our ongoing combined ultrastructural/immunocytochemistry/genetic studies of the pathogenic yeast *C. neoformans*.

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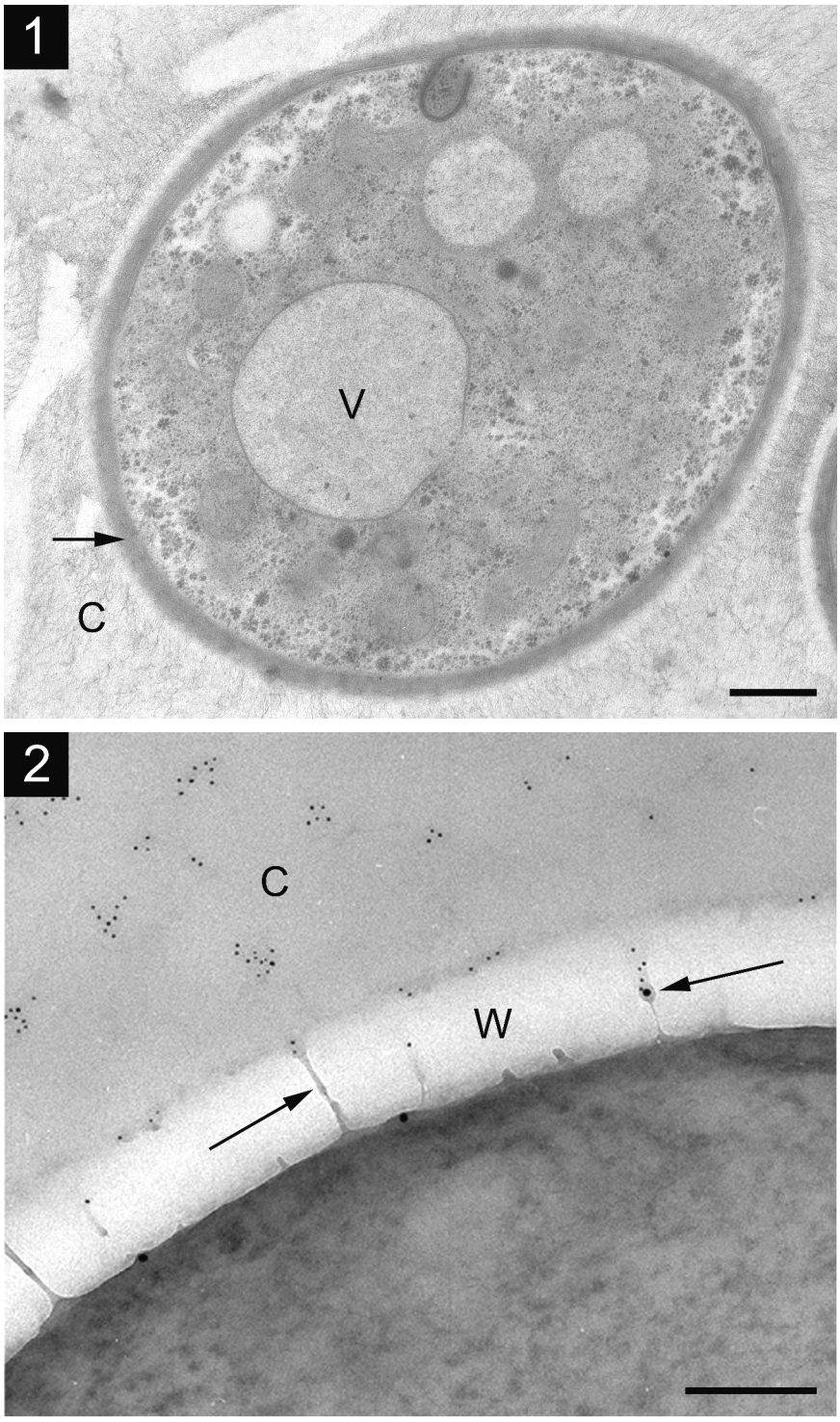

Fig. 1. A well-preserved *C. neoformans* cell. The capsule (C) extends out from the cell wall (arrow). A large vacuole (V) is indicated. Bar equals 0.5 μm.

Fig. 2. Surface antigens, indicated by large and small gold particles, leave the cell via channels (arrows) through the cell wall (W). The capsule (C) is not apparent, but is decorated with the smaller gold particles that denote the location of the capsular molecule 2H1. Bar equals 0.2 μm.

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