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NOTES

Laccase Expression in Murine Pulmonary *Cryptococcus neoformans* Infection

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Cryptococcus neoformans **laccase expression during murine infection was investigated in lung tissue by immunohistochemistry and immunogold electron microscopy. Laccase was detected in the fungal cell cytoplasm, cell wall, and capsule in vivo. The amount of laccase found in different sites varied as a function of the time of infection.**

Cryptococcus neoformans is a fungal pathogen that affects primarily patients with impaired immunity (11). *C. neoformans* has several characteristics that allow it to survive in mammalian hosts and cause disease; these include the ability to grow at 37°C, the production of a polysaccharide capsule, expression of laccase, urease, and phospholipase, and mannitol synthesis (1, 2, 5, 13–15). The relative importance of these fungal attributes is not understood, but the presence of the capsule and laccase and laccase-catalyzed melanin pigment production are considered major virulence factors.

Laccase activity in *C. neoformans* is associated with virulence (8, 14). Laccase has been proposed to contribute to virulence through melanin synthesis, iron acquisition, interference with oxidative burst, and facilitation of extrapulmonary dissemination (7, 10, 12, 16). Laccase was reported to be associated to the cell wall through a reducible bond (17). Despite extensive studies in vitro, only scant information is available on laccase expression in vivo. In vivo transcription of laccase was determined by reverse transcriptase PCR amplification of *CNLAC1* from rabbit cerebrospinal fluid (14). Given the importance of this virulence factor and the lack of conclusive evidence for protein expression in vivo, we generated monoclonal antibodies (MAbs) to laccase (17) and used them to study laccase expression in vivo and in vitro.

BALB/C and C57BL/6 mice were injected intravenously in the tail vein with 10^4 yeast cells of *C. neoformans* strain 24067. Serum analyzed by enzyme-linked immunosorbent assay revealed no antibody to laccase or capsular polysaccharide at day 28 of infection. The negative serological result suggested either that laccase was not made in vivo or that it was expressed in insufficient quantities to stimulate an antibody response. Furthermore, multiple peritoneal immunizations with laccase in adjuvant conferred no protection to mice challenged intravenously with *C. neoformans*, despite the presence of antibody titers to laccase (data not shown). Given that a negative serological result was inconclusive, we investigated whether laccase was expressed in vivo by detecting the protein in tissue.

C57BL/6 mice (6 to 10 weeks old) were infected intratracheally with 10^4 cells of *C. neoformans* strain 24067 and sacrificed after 8, 16, 24, or 48 h or 7, 14, and 28 days. Mice sacrificed at 8 and 16 h were infected with 10^6 cells. After the desired time interval, lungs were removed, fixed, processed, and sectioned for immunohistochemistry or immunogold labeling as previously described (3). Sections were labeled with either laccase-binding MAb G3P4D3 or isotype match control Ricin 45 followed by incubation with goat anti-mouse immunoglobulin G1 (IgG1) conjugated to horseradish peroxidase. The presence of laccase was demonstrated by developing the tissue sections with diaminobenzidene for 8 to 13 min. Immunohistochemical staining showed that some cryptococcal cells were strongly stained by laccase-binding MAb, consistent with laccase expression. Staining was very strong around the capsule, within macrophage vesicles, and in surrounding tissues (Fig. 1c). Hence, we investigated laccase expression in tissue using immunogold electron microscopy. Ultrathin sections 70 to 80 nm thick were prepared in nickel grids, treated with aqueous 10% H_2O_2–sodium periodate, and labeled with MAb G3P4D3 or irrelevant control MAb 293. Localization of laccase in tissue was determined by labeling with a biotinylated goat anti-mouse IgG1 followed by incubation with streptavidin conjugated to 10-nm-diameter gold particles (1:30). This method confirmed binding of the MAb G3P4D3 to *C. neoformans* laccase in vivo and revealed cell-to-cell variability in binding (Fig. 1d to h). The specific labeling and distribution of gold particles in 10 cells from different fields for each time were quantified as an indicator of laccase expression, and the data

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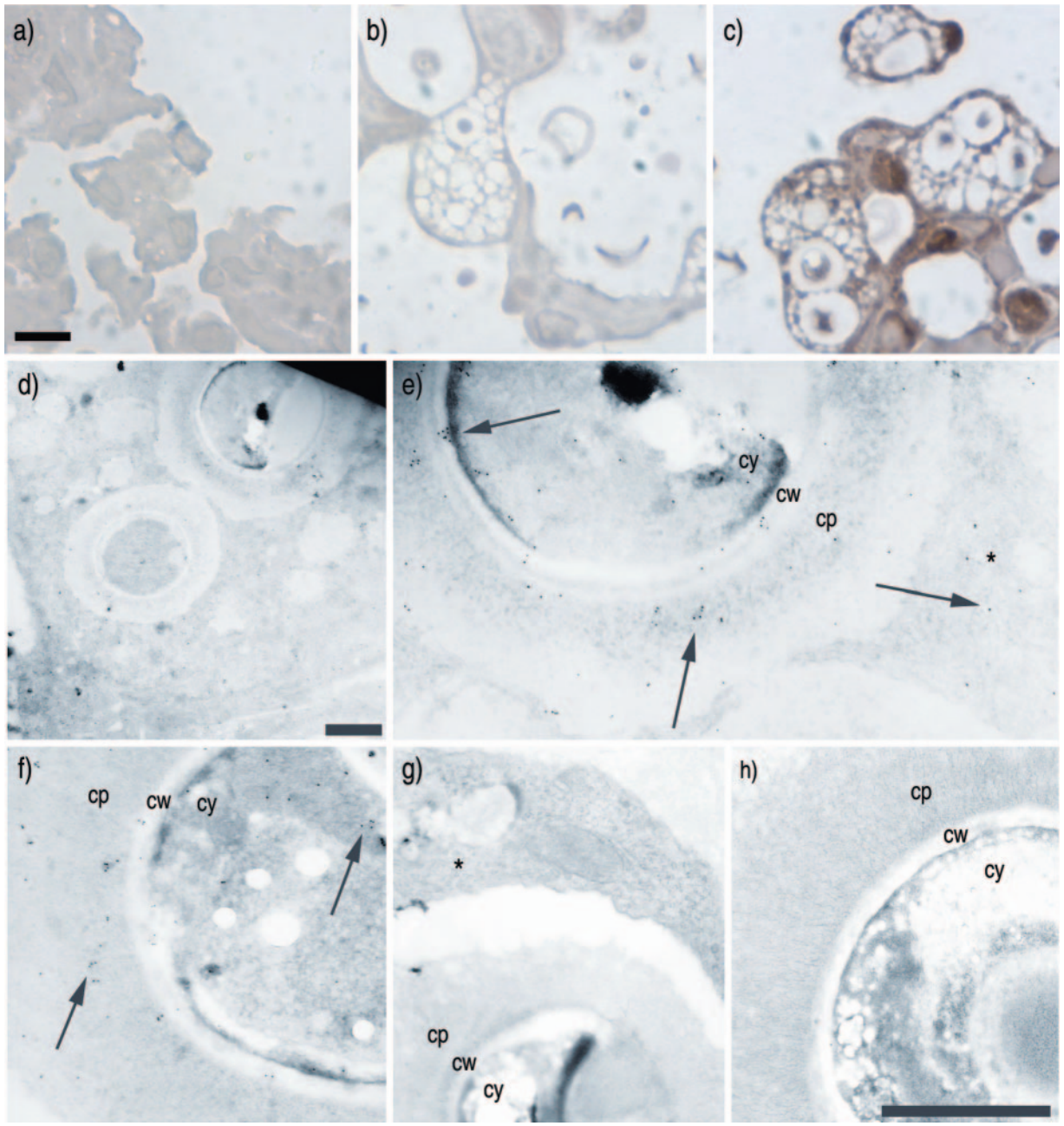

FIG. 1. Detection of laccase by immunohistochemistry in infected lung tissue 7 days after infection. Uninfected tissue probed with IgG1 MAb G3P4D3 (a), infected tissue probed with isotype match control MAb Ricin 45 (b) or MAb G3P4D3 (c). Electron microscopy of a C57BL/6 lung infected with *C. neoformans* (d), yeast cells labeled with MAb G3P4D3 at day 7 (e) and 16 h (f). Panels g and h were yeast in lungs at day 7 labeled with isotype-matched irrelevant control MAb 293. Arrows indicate gold particle labeling, cytoplasm (cy), cell wall (cw), capsule (cp), and host cell (*). Scale bars, 10 μm (a-c) and 1 μm (d-h).

were analyzed by the Kruskal-Wallis test using Prism version 4 (GraphPad Software, Inc., San Diego, CA). At 16 h, gold particles were equally distributed among the cytoplasm, cell wall, and capsule, indicating the presence of laccase in all three areas (Fig. 2). At 24 h, there was a significant ($P < 0.001$) increase in laccase present in the capsule compared to 8 h, showing a maximum average value of 24.4 gold particles per cell. In contrast, laccase labeling in the cytoplasm decreased. After 24 h, a significant decrease ($P < 0.001$ for day 7 and $P < 0.01$ for day 14) of laccase expression was observed, and the relative distribution shifted such that it was found predominantly in the capsule instead of the cytoplasm. Although some fungal laccases are secreted (9), this result is significant for *C. neoformans* because laccase was previously considered to be a cell wall-associated virulence factor (17). No significant labeling was observed with MAb 293.

We considered the possibility that the reduction in laccase expression reflected *C. neoformans* killing by host defense

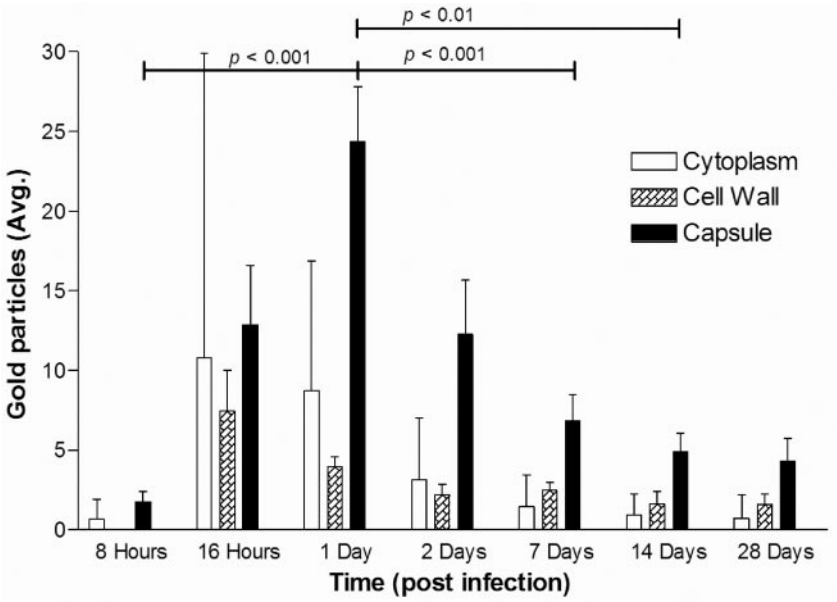

FIG. 2. Quantification and distribution of laccase labeling as monitored by immunogold labeling of lung tissue taken during the course of infection. The figure represents the pooled data of two independent experiments with similar results. Error bars represent the standard deviation of the mean.

mechanisms. Therefore, we measured fungal burden as a function of time. The highest expression of laccase was observed at day 1 when the number of fungal burden was the lowest in the lung. Moreover, CFU increased after day 1 whereas laccase decreased, suggesting that reduction in laccase expression was not due to killing of the organism.

Our results suggest that laccase is highly expressed early in infection, where it could potentially contribute to virulence by catalyzing the formation of melanin precursors and by protecting the yeast against alveolar macrophages by acting as an iron scavenger (10). Interestingly, the observation that laccase localization shifts and that the amounts decrease coincides with the observation that *C. neoformans* is mostly found intracellularly in macrophages during the first 8 hours of infection (4). The mechanism by which a reduction in laccase expression is regulated in vivo is unknown, but potential explanations include reactions to progressive changes in the type of cells found in the inflammatory response and/or metabolic changes associated with *C. neoformans* adaptation to survival in vivo. Down-regulation of laccase at later stages of infection could also be attributed to repression by temperature (6). However, thermal regulation would not explain the rapid rise in expression measured in the first 24 h of infection. Likewise the reduction in laccase expression based on reduced viability of *C. neoformans* is unlikely, since fungal burden as measured by CFU remained stable during the first 3 weeks of infection.

The demonstration that laccase is made in vivo and that some of the enzyme is released from the cell wall could have important implications for our understanding of the mechanism(s) by which laccase contributes to virulence. The release of laccase from the cell wall implies the possibility that laccase acts at a distance and may contribute to virulence by interfering with oxidative microbicidal mechanisms or iron acquisition from host cells infected with *C. neoformans* as well as neighboring cells. Further analysis of laccase expression will be necessary to understand the regulation and mechanism of action of laccase in vivo.

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