

ULTRASTRUCTURE OF *CRYPTOCOCCUS NEOFORMANS*

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Abstract. The ultrastructure of *C. neoformans* was studied in two cases of cutaneous cryptococcosis. Extracellular and intracellular fungi were observed. Their capsules and clear zones were thick and distinct, but their cytoplasmic organelles were not obvious. In macrophages, a few stages of fungal degeneration could be observed. It could not be ascertained whether the changes were caused by anti-fungal drugs or by an influence by host macrophages. The ultrastructure of the yeast cells *in vitro* appeared to be that of degenerated cells because of a long culture period.

Key words: Cutaneous cryptococcosis; *C. neoformans*; Ultrastructure

The fine structure of *Cryptococcus neoformans*, cultured *in vitro* on agar, has been reported by several authors (1, 3, 6-8, 10, 14). Noble & Fajardo (11), however, studied material *in vivo* from a patient with primary cutaneous cryptococcosis.

In previous papers (12, 13), one of us described two cases of cryptococcosis. In this study, the ultrastructure of *C. neoformans* taken from these 2 patients *in vivo* was observed. The process of degeneration of yeast cells in the tissue was discussed. The fine structure *in vivo* was also compared with that *in vitro*.

MATERIALS AND METHODS

The clinical pictures of 2 patients with cryptococcosis, one with secondary cutaneous cryptococcosis, one with a subcutaneous cryptococcal abscess caused by primary osseous cryptococcosis, have already been reported (12, 13).

Material for electron microscopy was biopsied. The specimens from the first patient were obtained from the granulomatous wall of a thumbtip-sized subcutaneous nodular abscess on the flexor side of the left lower leg. Biopsies from the second patient were removed from the granulomatous wall of an abscess in the midsternal line. These tissues were fixed with cold 0.1 M cacodylate-buffered 2.5% glutaraldehyde, pH 7.2, for 5 hours, and post-fixed with 1% osmium tetroxide in the same buffer for one

hour. The tissues were dehydrated in a series of alcohols, and embedded in Epon 812.

Material obtained from the biopsy of the first patient was cultured on a Sabouraud agar plate at 37°C for 4 weeks and the colonies transferred to cold 0.1 M cacodylate buffered 2.5% glutaraldehyde, pH 7.2. After 3 hours, the material was centrifuged and the pellet washed with the same buffer. After one hour of post-fixation with 1% osmium tetroxide it was dehydrated in a series of alcohols and embedded in Epon 812.

Ultrathin sections were cut with a Porter-Blum ultramicrotome, stained with uranyl acetate and lead citrate, and observed in a Hitachi HS-8 electron microscope.

RESULTS

When the biopsy was taken, the first patient had been treated with 5-Fluorocytosine (5-FC) and Amphotericin B (AMPH), in total 380 g and 140 mg, respectively. In the specimens, only free extracellular fungi were found, whose diameter ranged from 3000 to 12000 nm (Figs. 1, 2). Around these extracellular yeast cells, no limiting membranes were observed. Their microfibrillar capsules and translucent clear zones were thick and distinct. Several of the fungi were surrounded by a non-structural space separating them from the connective tissue. In such cases, the cell walls were about 100 nm thick and had high electron density. The inside of the yeast cell bodies was homogeneous, and no cytoplasmic organelles were seen.

Before the biopsy was taken, the second patient had been treated with 5-FC, in total about 400 g. In contrast to the first case, only intracellular fungi were observed (Figs. 3, 4, 5). Not only one, but even two or three yeast cells were found in the host macrophages. The capsules of these intracytoplasmic fungi were surrounded by a 10 nm thick limiting membrane. The clear zones were thick and most evident. However, there were several different clear zones and capsular components as seen in Figs. 3 and 4B. Figs. 4A and 5B show only these

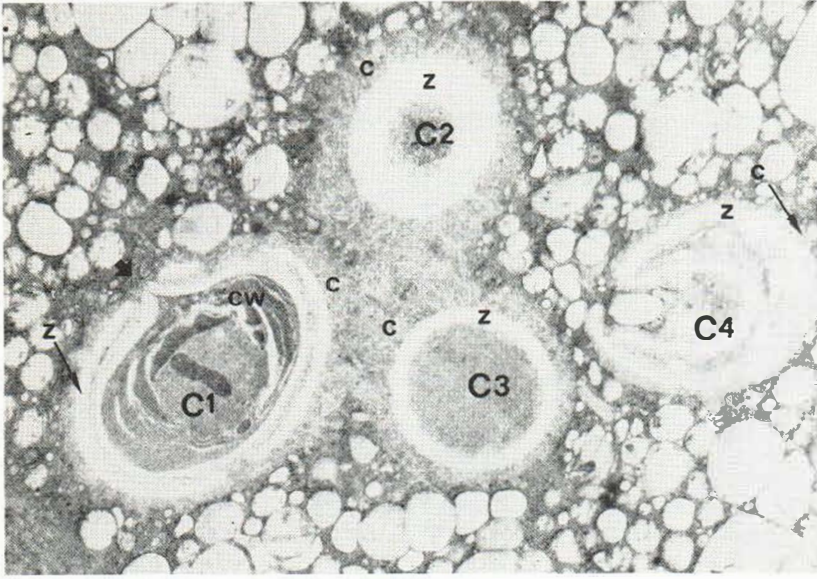


Fig. 1. First case. Only extracellular fungi were found (C1-C4). C1, sectioned obliquely, shows budding (wide arrow) and a cell wall (cw) which displays an onion skin appearance. c, capsule; z, clear zone. $\times 23\ 400$.

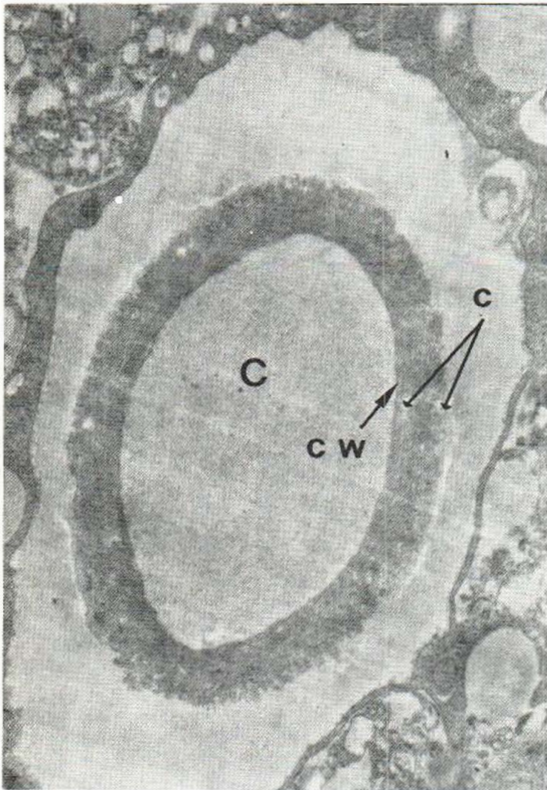


Fig. 2. Extracellular fungus (C) has a thick cell wall (cw) and capsule (c). Arrows show thickness of capsule. No cytoplasmic organelles are seen. A non-structural space is situated between the fungus and the surrounding tissue. $\times 60\ 000$.

components, the inside of the fungi being replaced by cytoplasmic components of the host macrophage, while in other places almost half of the capsules or only very small fragments are left (Fig. 5). These phenomena probably show the progress of degeneration of the fungi in macrophages.

The fungi from the first patient still maintained sufficient activity to grow on Sabouraud agar plates in spite of the above-mentioned treatment (Fig. 6). Outside the yeast cells, capsules were seen consisting of microfibrils, 2-4 nm in diameter and 6 000-12 000 nm long. In comparison with the fungi of the in vivo material, however, these capsules were very thin. A vacant area was situated between the plasma membrane and the cell wall. Details of cytoplasm and intracytoplasmic organelles were not obvious. The findings show that the yeast cells were on their way to degeneration, 4 weeks being a rather long period to observe details of cultured fungi.

DISCUSSION

In the present study, the cultured yeast cells on Sabouraud agar plates showed figures different from those reported previously (1, 3, 6-8, 10, 14), probably because of the long period of culture. However, our findings were identical with those of degenerated cryptococci, i.e. the vacant area between the plasma membrane and the cell wall, the

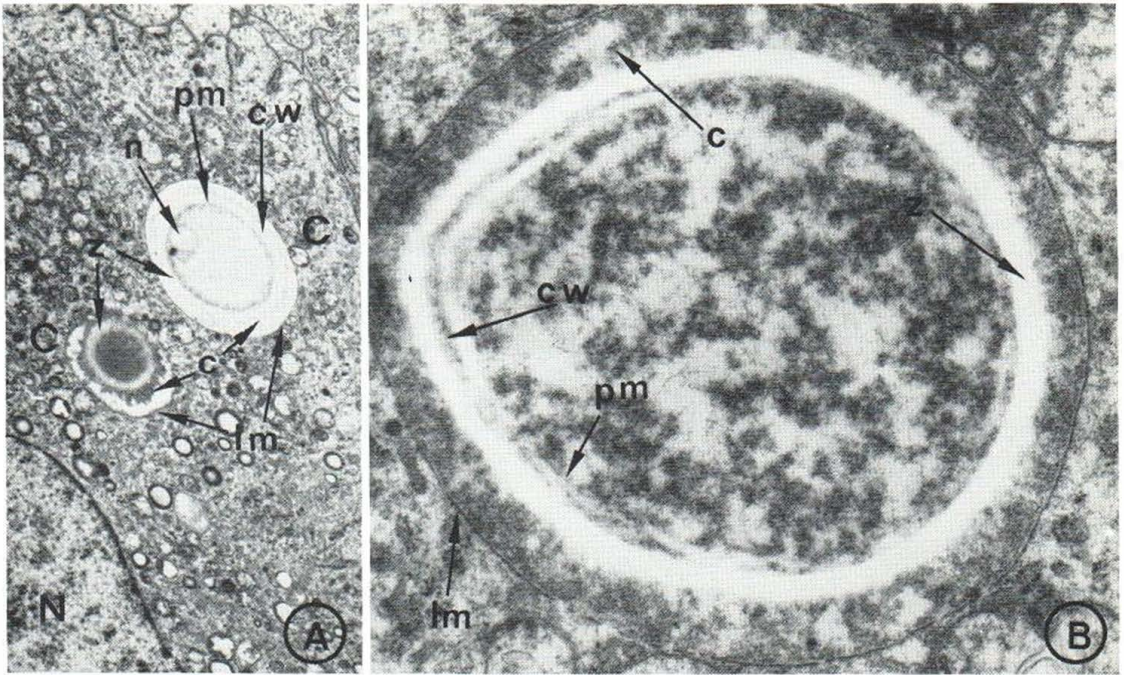


Fig. 3. (A) Second case. Only intracellular fungi (C) were found in macrophages. They were surrounded by a clear zone (z), a capsule (c), and a limiting membrane (lm). Some show a nucleus (n) and a plasma membrane (pm). N, nucleus of macrophage. $\times 15000$. (B) This interior of

an intracellular fungus is not characteristic. A plasma membrane (pm), a cell wall (cw), a clear zone (z) and a capsule (c) are recognized. The fungus is surrounded by a limiting membrane (lm). $\times 90000$.

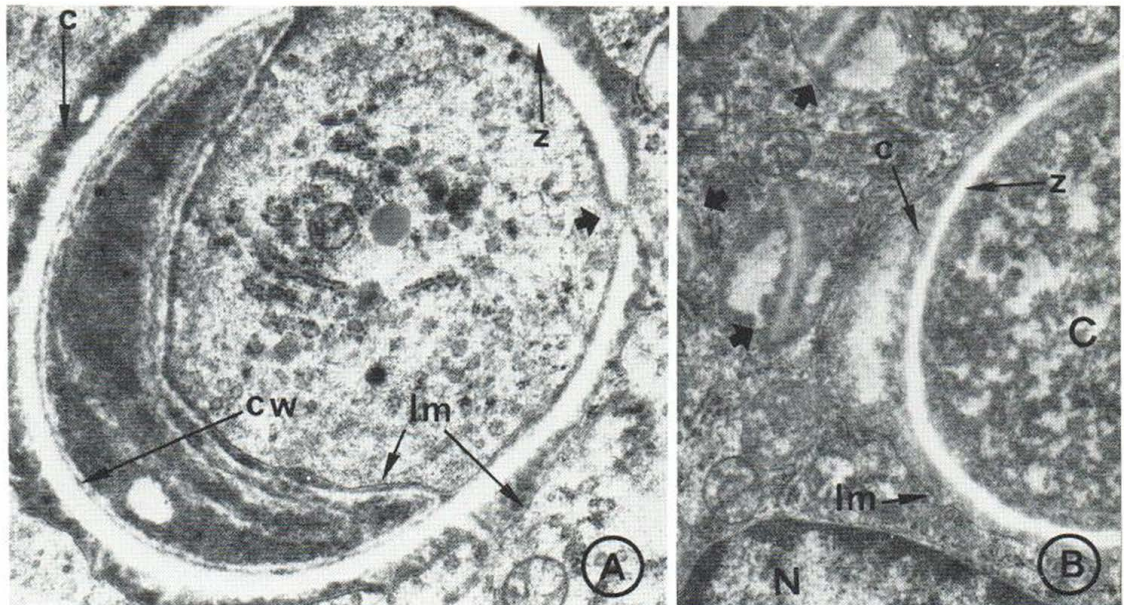


Fig. 4. (A) Intracytoplasmic organelles belonging to a host macrophage are seen in a cryptococcus. Part of the clear zone is broken (broad arrow). $\times 90000$. (B) Around the intracellular fungus (C), several pieces of clear zone

and capsule, surrounded by a limiting membrane show lysosome-like structures (broad arrows). N, nucleus of host macrophage. $\times 90000$.

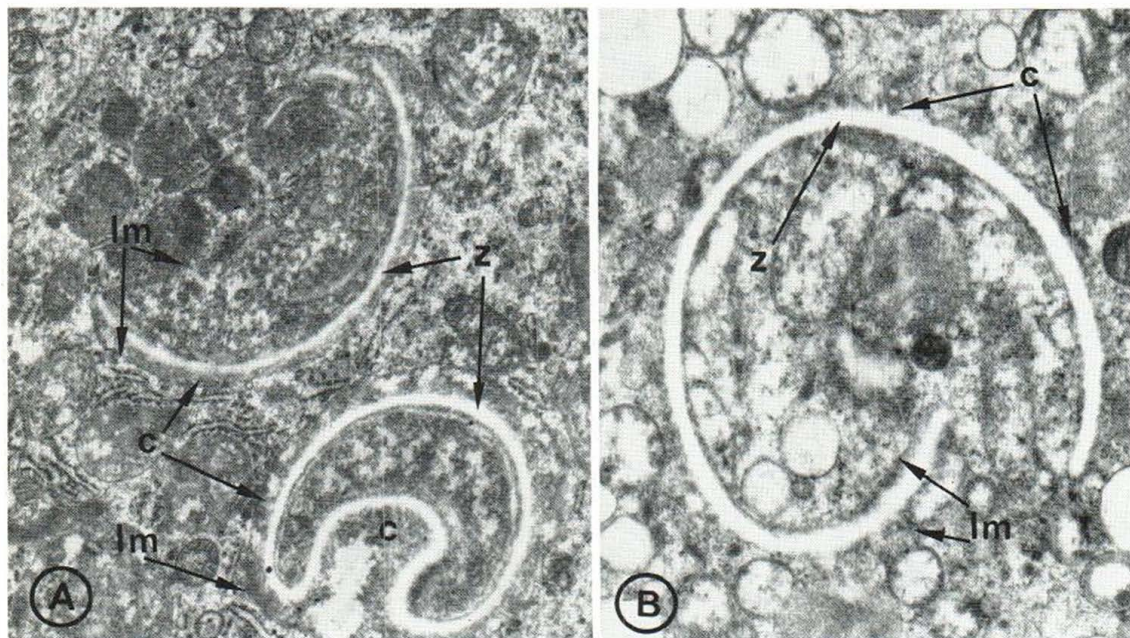


Fig. 5. (A) In a macrophage, a half part of a clear zone is surrounded by capsule and limiting membrane. $\times 30\,000$. (B) Only a clear zone with capsule and limiting membrane

is seen. Cellular fungal components are no longer observed. $\times 60\,000$.

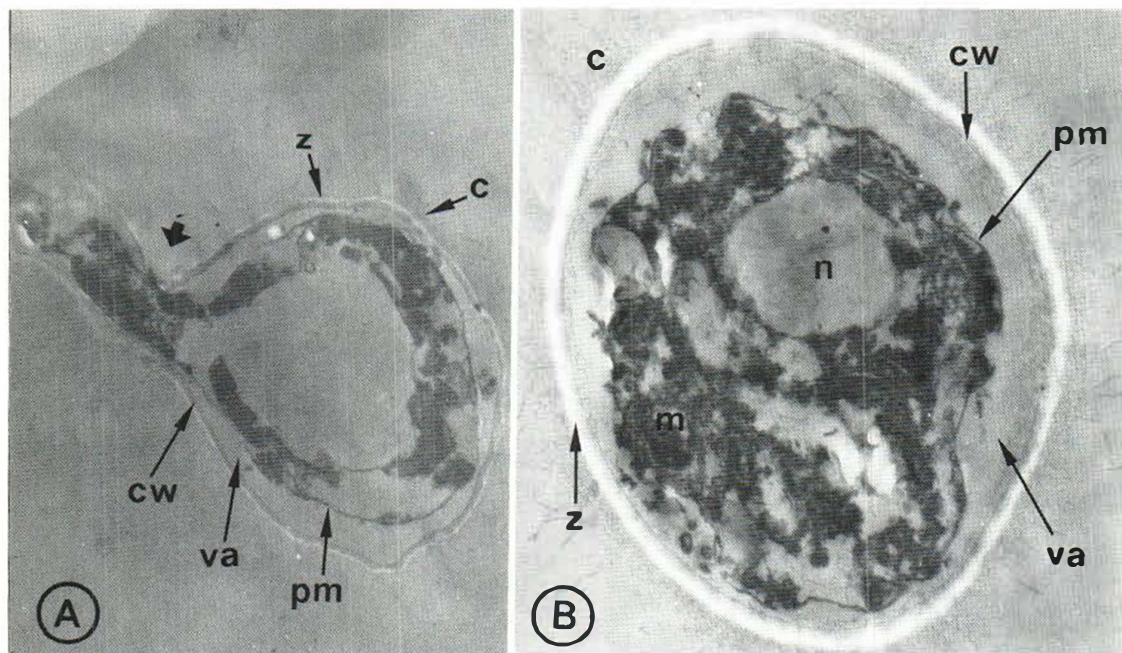


Fig. 6. Fungi from the first patient, cultured on Sabouraud agar plate, after 4 weeks. (A) A few filamentous capsule parts (c) and a very narrow clear zone (z) are seen. Between the cell wall (cw) and the plasma membrane (pm), a vacant area (va) is located. A broad arrow shows budding.

$\times 90\,000$. (B) Nucleus (n), mitochondria (m), clear zone (z) and filamentous capsule (c). A vacant area (va) is located between the plasma membrane (pm) and the cell wall (cw). $\times 90\,000$.

thin capsules and the indistinct intracytoplasmic organelles (1, 3).

Edwards mentioned that the thick capsules and distinct clear zones were seen in old yeast cells *in vitro* (3). We observed these phenomena even in tissues *in vivo*.

In the second case, there were various appearances of clear zones and capsular components. The cryptococci seemed to degenerate stepwise in macrophages. The authors could find no similar reports in the world literature. The clear zones, actually belonging to the fungal capsules, were suspected to represent chitin (4), a principal component of fungal exoskeletons. This polysaccharide probably protects the fungi from influences of their surroundings (1, 4). It is assumed to be natural that fragments of these capsular components were hard to change and were left as remnants in the cytoplasm of macrophages.

In the fungi, the anti-fungal drug 5-FC is metabolized to 5-Fluoro-uracil (5-FU) which inhibits the DNA synthesis, whereas 5-FC is not metabolized by the human body (2). AMPH causes structural changes in the fungi, being bound to membrane lipids with resultant leakage of intracellular materials, a decrease in cell biosynthetic function, and, eventually, cell death (5, 9). Noble & Fajardo (11) mentioned that fungi in which nuclei and cytoplasmic organelles are invisible can be seen in primary cutaneous cryptococcosis even before treatment with AMPH. Our findings after starting treatment might or might not be caused by the anti-fungal drugs or the defence mechanisms of the host.

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