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Fine Structure of *Cryptococcus neoformans* Grown In Vivo as Observed by Freeze-Etching

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Cryptococcus neoformans grown in the parasitic state was observed by the freeze-etching technique and was compared with that grown on culture media. Unlike other yeasts, this organism grown in vivo is very often devoid of the "ordinary" invaginations. The membrane of the cell grown in vivo was almost free from concavity and convexity except for many round depressions which represent the surface view of paramural bodies. Some of the paramural bodies were found to be multivesicular systems. Most were spherical invaginations containing a single vesicle or its ghost remaining after secretion of the vesicles. In clear contrast to the cell grown in vitro, the in vivo cell contained a great number of vesicles in the cytoplasm. These seemed to show high-secretion activity in *C. neoformans* grown in the parasitic state. On transfer from in vitro to in vivo, this organism enlarged the cell wall, capsule, and cell body. The appearance of a large vacuole, accumulation of storage organelles, and the existence of rodlike structures, seemingly lipid deposits, were also noted in the cytoplasm of the cell grown in vivo. The meaning of these results as well as the mode of capsular production are discussed.

It is well established that pathogenic organisms grown in a parasitic state are different in many respects from those grown on culture media. *Mycobacterium* isolated from infected mouse tissues exhibit reduced oxidation capacity because of deficiency of cytochromes (6, 13). *Staphylococcus aureus* grown in vivo, on the contrary, shows a higher state of oxidation activity and produces far more hemolysin, leucocidin, and tissue-attacking enzymes (1, 4). Most pathogenic fungi show dual aspects of the morphology of fungi in their saprophytic and parasitic states (8). On transfer from in vitro to in vivo, *Cryptococcus neoformans* does not undergo radical changes in morphology as do other pathogenic fungi. It remains in yeast form, but increases cell and capsule size. Because of the moderate morphological changes of this organism, it is easy to compare and relate them to biological activity. It was reported by cytochemical studies that *C. neoformans* grown in vivo shows higher activity of alkaline phosphatase and other enzymes than that grown in vitro (7, 16). No paper, however, seems to have appeared comparing the fine structures of this organism grown in vivo with those grown in vitro.

We have already studied this organism grown in vitro by using a freeze-etching technique (15). This paper describes the fine structure of *C. neoformans* grown in vivo and compares it with that of cells grown in vitro. The mechanism of capsule production also is discussed.

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MATERIALS AND METHODS

Organisms. *C. neoformans* strains Okuno and IFO 608 (abbreviated as 608) were used as in vitro (15). They were grown on nonglycerol medium for 2 days at 37 C before inoculation into mice.

Experimental animals. Female mice (DDD strain) about 4 weeks old and weighing, on the average, 20 g were used. Usually, mice received 5 mg of hydrocortisone acetate subcutaneously 2 days prior to injection of *C. neoformans*. About 10,000,000 organisms were injected intraperitoneally. The in-

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fected mice were killed 3 to 14 days later, and the lungs or brains were dissected. The dissected tissues were fixed with 5% glutaraldehyde containing 0.067 M phosphate buffer, pH 7.4, for 2 to 3 h at room temperature, and then were immersed in 20 to 40% glycerol for 3 to 5 days at 4 C. Other methods were the same as previously described (15). All the figures in this paper show the freeze-fractured Okuno strain.

RESULTS

When *C. neoformans* was transferred from in vitro to in vivo, the size of cell body and capsule increased. The degree of change seemed to vary with host tissue and strain. In the brain, the cell and capsule increased only slightly in size. Generally speaking, in tissue where many cells were found, the cell and capsule were rather small. Probably rapid growth is not favorable for large cell and capsule production. In the lung and spleen, large cells with conspicuous capsules appeared. The largest cells of Okuno strain reached more than 8 μm in diameter, although the cell size was not as uniform as in vitro (15). Similar results, to a lesser extent, were obtained in the 608 strain.

Capsule. As can be seen clearly in Fig. 1, the freeze-fracture did not reveal capsular structures. The capsule was recognized as such by its location, limited by the wall inside and host tissue outside.

Cell wall. The wall looked like that of in vitro cells but was thicker. In cells larger than 6 μm in diameter, the walls were about 300-nm thick. Accumulation of particles of about 20 nm in size also were seen in the outer layer of the wall of about 80-nm thickness. Interestingly, some of the particles seemed to be excreted into the capsule (Fig. 1 and 6).

Cell membrane. Features of the cell membrane changed markedly on transfer from in vitro to in vivo. Wrinkles were scarcely found in vivo but were abundant in the in vitro cells. In each cell membrane, "ordinary" invaginations were very often lacking, but when found they were short and straight as shown in Fig. 2. The most distinct structures of the cell membrane were round depressions of 40 to 200-nm width and of varying depth (Fig. 3). The typical round depressions did not bear cell membrane particles, whereas the smaller and shallow ones did. The round depressions were calculated to be as many as 1000 in the total cell membrane of a single cell as shown in Fig. 3.

Paramural body. Two types were observed as in vitro cells. One was a spherical invagination of 100 to 150 nm in size containing a single vesicle about 50 nm in diameter. Shallow, round depressions, found in great quantity in the cell membrane, are thought to be the ghosts of spherical invaginations remaining after secretion of the vesicle (15). The other was a baglike structure of 200 to 600 nm in diameter. Figure 4 shows the multivesicular paramural body, probably containing 6 to 10 vesicles.

All the membranes of the paramural bodies observed were free of particles.

Cytoplasmic organelles. The cytoplasm of *C. neoformans* grown in vivo contained a great number of vesicles as shown in Fig. 6. Vesicles of 30 to 70 nm in diameter, about the same size as found in the spherical invaginations, were distributed throughout the cytoplasm. In a cross-fractured cell, 100 to 200 vesicles were seen. Most of the vesicles took the spherical shape. Because only those vesicles within about a 50-nm distance from the cross-fractured plane were visible, we can calculate that one cell body contains up to 20,000 vesicles.

Structures with multilayered shells, known as storage organelles containing polar lipids (10), developed in cells grown in vivo. Their size and number were the same as or greater than those in the stationary-phase cells grown in vitro. Noteworthy are the rodlike structures which were surrounded also by multilayered shells. As shown in Fig. 6, they were about 60 nm in diameter and more than 400 nm long.

The larger cells grown in the tissues generally possessed only one large vacuole of 3 to 5 μm in diameter. Sometimes rodlike structures were seen in it. A large vacuole containing several small vacuoles and vesicles was also found, as is shown in Fig. 5.

The nucleus was similar in size and shape to that of in vitro cells. Generally speaking, the in vivo cells possessed more cytoplasmic organelles, which were 100 to 500 nm in size, than in vitro.

DISCUSSION

On transfer of *C. neoformans* from in vitro to the parasitic state, the cell wall, the capsule, and the cell body enlarge; this seems to be the result of the slow rate of cell division of this organism when grown in tissues of mice and relatively active metabolism, such as synthesis of the wall, capsule, and cytoplasmic material. The large vacuole and the accumulation of storage organelles in the cytoplasm also suggest a lower state of multiplication of the organism in the tissues, whereas the large number of vesicles found in the cytoplasm and round depressions in the cell membrane seem to show high secretion activity. The large cells, grown in vivo, also have rodlike structures with a

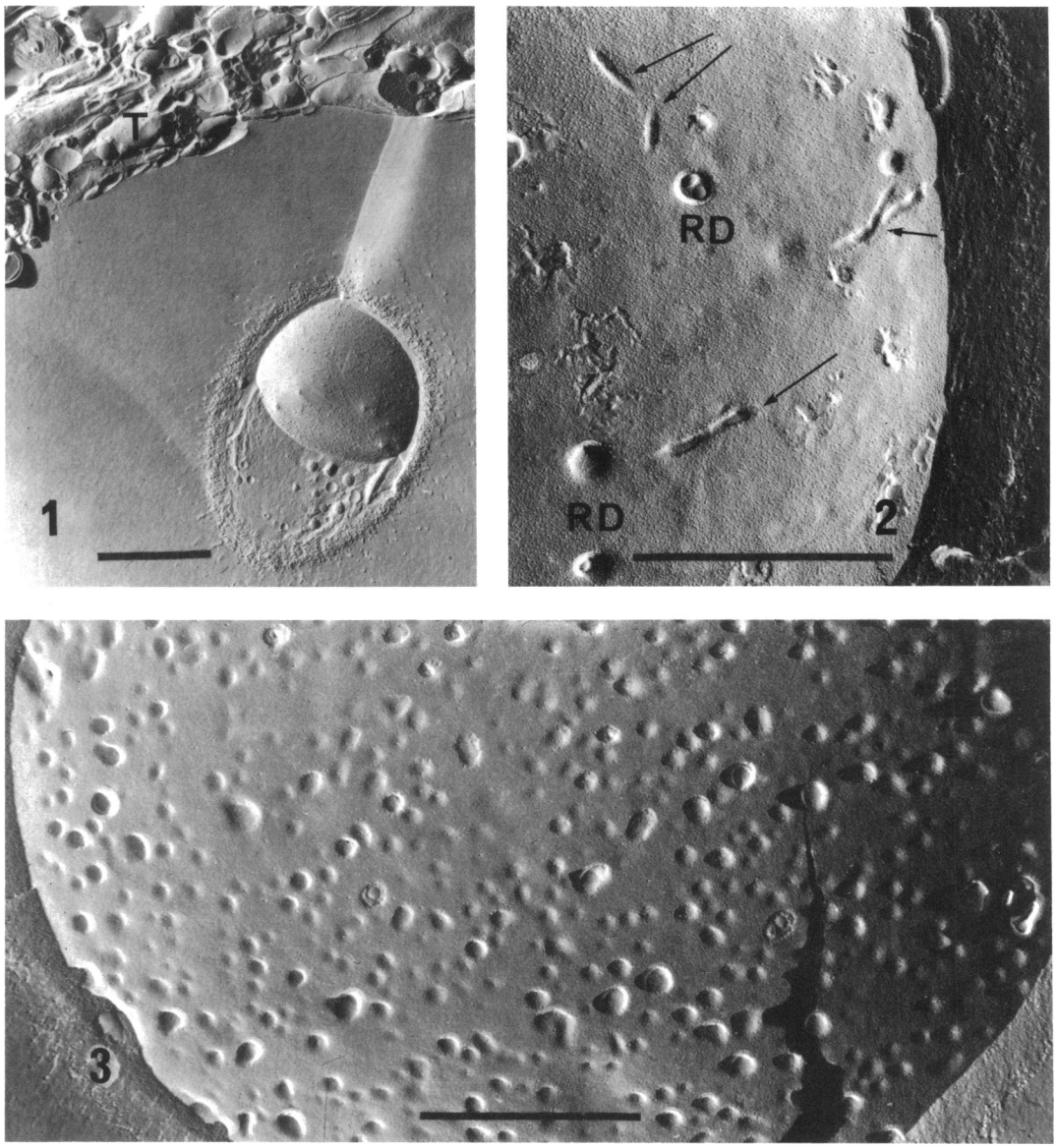

FIG. 1. *The capsule of C. neoformans surrounded by the tissue (T) of the brain. Note that the wall particles appear to be excreted into the inner part of the capsule. The bar in this and in all subsequent figures indicates 1 μm.*

FIG. 2. *Outside view of cell membrane showing short and straight invaginations (arrows) and round depressions (RD). This and all the subsequent figures represent C. neoformans from the lung lesion.*

FIG. 3. *Inside view of cell membrane showing many round protrusions, measuring 40 to 200 nm in size, which actually are depressions of the membrane when viewed from outside. This cell was also found in the lung. Five protrusions were sliced, and in each protrusion one or two vesicles about 50 nm in diameter are seen. No wrinkles nor "ordinary" invaginations of the cell membrane were found.*

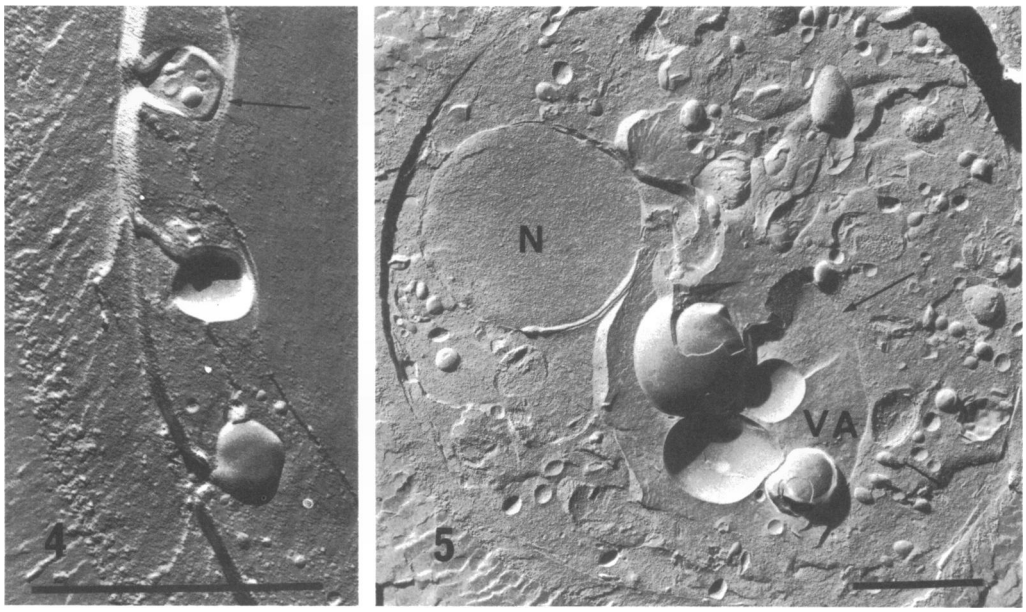

FIG. 4. *A cross-fractured baglike paramural body showing multivesicles (arrow). See also the outside view of the paramural body, which does not bear particles.*

FIG. 5. *Cross-fractured cell showing a large vacuole (VA) which contains several vacuoles and vesicles. The membrane of the large vacuole (arrow) is not clearly seen in its total boundary, but the vacuole can be discriminated from the other cytoplasm because the former is sparse in particles. See also the profile of the nucleus (N) and the many organelles and vesicles.*

multilayered shell. They seem to be deposits of cytoplasmic material and may consist of lipids of polar nature, judging from the appearance of the multilayered shell (10).

Interestingly, the invaginations of the cell membrane in the parasitic state, if any, take the short and straight form as do those of other yeasts, whereas they are long and curved in the cultured state.

Vesicles of 30 to 70 nm in diameter are abundant in the cytoplasm of in vivo cells. Cytochemical studies have already revealed that *C. neoformans* grown in vivo exhibits a higher enzyme activity than in vitro (16). Liu reported that alkaline phosphatase exists in the capsule as well as in the cell body when grown in vivo, but not in vitro (7), which shows the induction and secretion of the enzyme in the parasitic state. Thus, some of the vesicles found so frequently in in vivo cells may be involved in secreting enzymes such as alkaline phosphatase or protease, as is the case in hyphae of *Neurospora crassa* grown in a protein medium (9).

It has been suggested that the wall material is contributed by the fusion of vesicles with the cell membrane and the consequent release of their contents to the wall (11). Heath et al. showed that the vesicles of some fungi contain cell wall precursor (5). Van der Woud et al. isolated from a germinating plant secretory vesicles containing polysaccharide which were similar in sugar composition to those of the hot-water-soluble fraction of the wall (17). Although the cell wall formation itself could not account for the existence of such a large number of vesicles in *C. neoformans* grown in vivo, capsule formation could be due to an overproduction of some wall materials (12, 14). Cadmus et al. reported in the case of *C. laurentii* conversion of 30 to 35% of glucose in the media to extracellular polysaccharide (2). It is probable that this large amount of capsular material is synthesized in and released via the vesicles or the baglike paramural bodies.

Edwards et al., by using a thin-sectioning technique, reported that a halo of 40 nm in width is located in this organism between the cell wall proper and capsule (3). They suggested that because of its uniform width and sparse content of microfibrils, it is a zone of capsular polysaccharide synthesis. In this connection it is very interesting that the outer layer of the wall, 40 to 80 nm thick, was filled with particles about 20 nm in diameter, which were different from the cell membrane particles

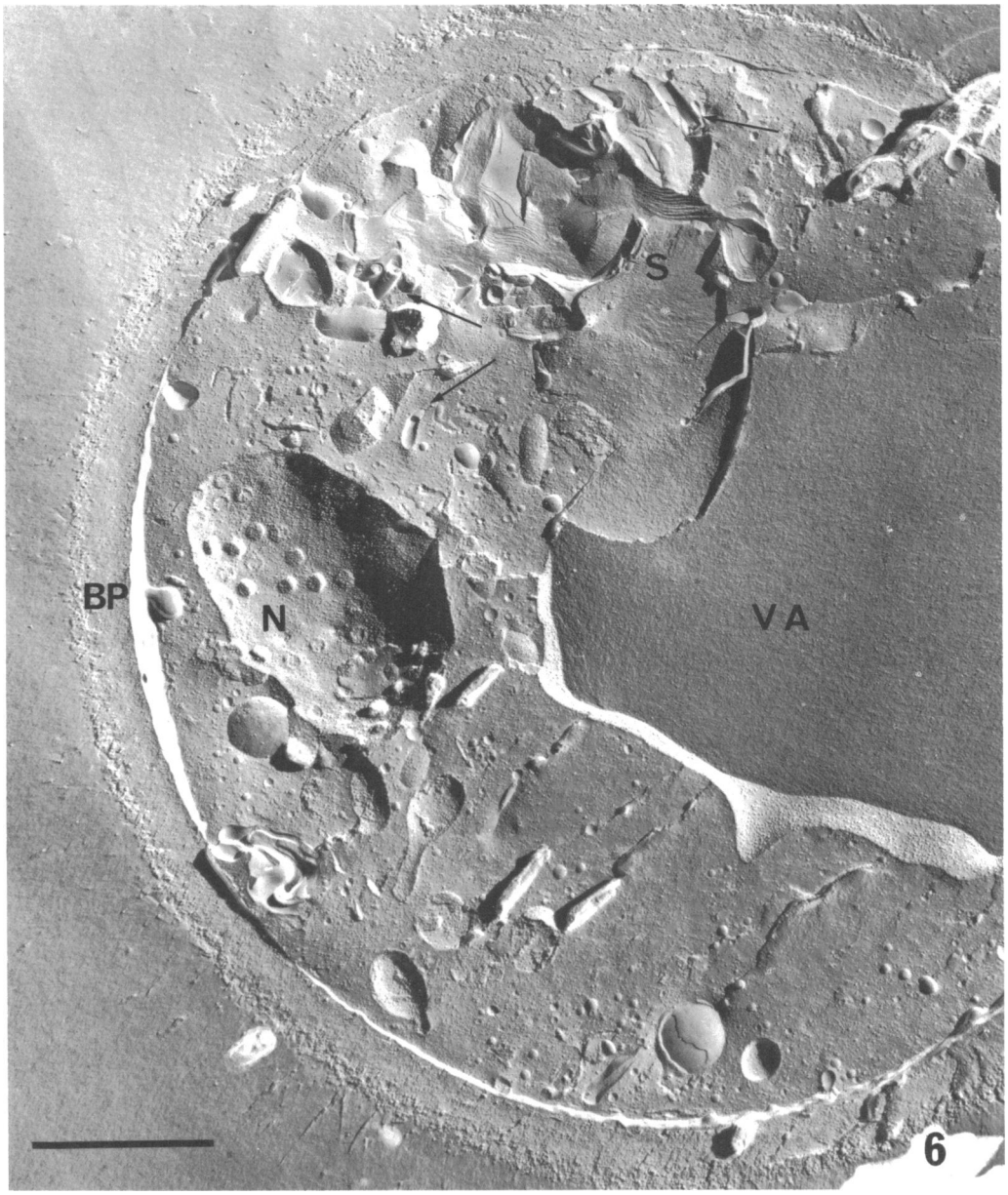

FIG. 6. *Cross-fractured cell showing many small vesicles, a storage organelle (S), rodlike structures (arrows), inside view of nucleus (N) with nuclear pores, large vacuole (VA), and baglike paramural bodies (BP). More than 200 vesicles, 30 to 70 nm in size, can be seen.*

in size. Some wall particles are excreted into the capsule. Accumulation of wall particles could also be found in other species of *Cryptococcus* but not in other genera of noncapsular yeasts studied (*unpublished data*).

Although more studies are needed to clarify the mode of capsular production, it can be suggested that the precursors of capsular material are synthesized in the vesicles or in the baglike paramural bodies and that they are secreted to the cell wall. At the outer layer of the wall the precursors are polymerized to the microfibrils of the capsule. The wall particles appear to be involved in the latter process.

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