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Ultrastructural study of *Cryptococcus neoformans* by quick-freezing and deep-etching method

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Abstract. The three-dimensional ultrastructure of *Cryptococcus neoformans* was studied by quick-freezing and deep-etching (QF-DE) method. *C. neoformans*, strain CDC551, was cultured on agar. The viable yeast cells (10^7 cells) were inoculated into each mouse from the tail vein. Three weeks after the inoculation, the brains of the mice were perfused with fixatives, quickly frozen, freeze-fractured, deeply etched and rotary shadowed with platinum and carbon. In addition, the viable cells of *C. neoformans* on agar were picked up and quickly frozen, and replica membranes were prepared as described above. The ultrastructure of *C. neoformans* was three-dimensionally demonstrated by the QF-DE method. The capsule was composed of fine meshworks of microfibrils (10–13 nm in diameter), which were directly attached to the cell walls. The capsule of the in vivo yeasts (yeast cells in the brain lesion) was thicker than that of the in vitro yeasts (yeast cells on agar culture). At the outer part of the cell wall, a particle-accumulating layer was observed. This layer in vivo was thicker than that in vitro. Occasionally, the yeast cells were ingested by phagocytes in the mouse brain. Although the cytoplasm of such yeast cells was destroyed, the capsular meshworks were well preserved. The ultrastructure of the capsule was the same both in cultured and phagocytized yeasts in the cystic lesions of the brains. This lack of morphological changes of the capsular meshworks suggests that they are resistant to the digestion by phagocytes. This stability of capsular structures may provide one of the important pathogenic factors in cystic lesions by *C. neoformans*.

Key words: Capsule, *Cryptococcus neoformans*, Deep-etching, Quick-freezing, Ultrastructure

Introduction

C. neoformans, an opportunistic fungal pathogen of the human being, is surrounded by a polysaccharide capsule which is capable of inhibiting phagocytosis by macrophages [1, 2]. Two types of lesions, granulomatous and cystic, are reported to be caused by this yeast [3]. Granulomatous lesions are formed in the organs rich in resident macrophages such as liver, spleen, while cystic lesions are formed in the organs in which they are scarce, such as brain [4]. Some ultrastructural studies of these yeast cells have been reported previously by using various techniques, revealing the general ultrastructures of the cytoplasm as well as that of the capsule [5–16]. However, these

conventional techniques tend to distort the native ultrastructure due to unavoidable chemical treatments.

The quick-freezing and deep-etching (QF-DE) method is one of the most suitable techniques for examining the filamentous structure at high resolution [17–19]. We have already reported the three-dimensional cytoskeletal organization of macrophages using the QF-DE method [20, 21]. In this study, we describe ultrastructure of unfixed fungal cells, especially that of the capsular structure. Moreover, the capsular ultrastructure of *C. neoformans* was compared between yeast cells grown in vitro (yeast cells on agar culture) and in vivo (yeast cells in the brain lesion), and a novel particle-accumulating layer was demonstrated to be localized between the capsule and the cell wall. To our knowledge, this is the first observation of *C. neoformans* by using QF-DE method.

Material and methods

Strains. Cryptococcus neoformans, CDC551 (serological type A), was used in this experiment.

Animals. 5-week-old male ICR mice (Shizuoka Experimental Animal Grower's Association, Shizuoka, Japan) were used in this experiment.

Preparation of lesions. (a) Cryptococcus suspension: The yeast was cultured on Sabouraud's dextrose agar at 30 °C for 3 days. The viable cells were suspended in sterile saline solution at a density of 1×10^8 cells/ml. (b) Inoculation of the suspension: Each mouse was inoculated intravenously with 0.1 ml of the cell suspension.

QF-DE method (a) In vivo (yeast cells in the brain lesion): Twenty one days after the inoculation, mice were perfused with 2% paraformaldehyde in 0.1 M phosphate buffer, pH7.4, (PB) for 5 min from the left ventricle of the heart under ethyl ether anesthesia. Brain tissues were cut into small pieces ($2 \times 2 \times 4$ mm) with razor blades, washed in PB to remove soluble materials from the cut surface of tissues, and fixed again with 0.25% glutaraldehyde in PB for 30 min [22]. They were rinsed in 10% methanol.

(b) In vitro (yeast cells on agar culture): Colonies on the agar were put on the plastic coverslips without fixation. Some colonies were suspended in saline solution, and centrifuged at 3,000 rpm for 10 min. The pellet was prefixed with 2% paraformaldehyde in PB for 5 min and fixed again with 0.25% glutaraldehyde in PB for 30 min. A small part of the pellet was placed on the plastic coverslips. These specimens were quickly frozen in an isopentane-propane mixture (−193 °C) cooled by liquid nitrogen [23] and fractured with a scalpel in liquid nitrogen. They were deeply etched in an Eiko FD-3S machine ($2–6 \times 10^{-7}$ Torr, −95 °C) for 15–20 min and rotary shadowed with platinum and carbon. Cellular components were dissolved in household bleach and replica membranes were picked up on copper grids.

Ultrathin section preparation. (a) In vivo (yeast cells in the brain lesion): Twenty one days after the inoculation, tissues were collected and fixed with 2.5% glutaraldehyde in PB at room temperature for 1 hr and washed with PB three times.

(b) In vitro (yeast cells on agar culture): The cells of *C. neoformans* were cultured as described previously and some cells were picked up and suspended in physiological saline solution. They were centrifuged at 3,000 rpm for 10 min. The pellet was fixed with 2.5% glutaraldehyde in PB at room temperature for 1 hr and washed with PB three times.

Both specimens were postfixed with 1% OsO_4 in PB for 1 hr, dehydrated in ethanol, and embedded in Quetol-812 (Nisshin EM Co., Tokyo, Japan). Ultrathin sections were cut with a Reichert Om-U3 ultramicrotome knife (thickness: 0.1 μm) and doubly stained with uranyl acetate and lead citrate. The replica membranes and ultrathin sections were observed with a Hitachi HS-9 electron microscope at 75 kV.

Table 1. The diameter of cells and the thickness of capsules of *C. neoformans* determined by the QF-DE method

	Diameter of *C. neoformans* excluding capsule (μm) (M ± SD)		Thickness of capsule (μm) (M ± SD)	
In vitro: $n = 11$ (Cells on agar culture)	3.78 ± 0.43	*	0.70 ± 0.11	**
In vivo: $n = 7$ (Cells in the brain lesion)	4.13 ± 0.25		1.50 ± 0.24	

M ± SD; Mean ± standard deviation.
*$p < 0.05$, **$p < 0.001$.

Statistical analysis. The replica electron micrographs were taken at random at the magnification of 20,000. The diameter of the yeast cells excluding the capsule and the thickness of the capsule were measured on the negative films. Statistical analysis were performed with Student's *t*-test for paired observations; comparison was made between in vivo ($n = 7$) cells and in vitro ($n = 11$) cells.

Results

The diameter of *C. neoformans*, excluding the capsule, was determined by the QF-DE method, is indicated in Table 1. There were small differences in size between in vitro and in vivo. The diameter of *C. neoformns* in vivo was a little greater than in vitro ($p < 0.05$).

Capsule. The thickness of the capsule of *C. neoformans* was greater in vivo than in vitro ($p < 0.001$) (Table 1). The capsular material was composed of fine microfibrils (10–13 nm in diameter calculated on the replica membrane). These microfibrils branched off and fused together to form a meshwork structure (Fig. 1). In replica membranes of the brain lesion, the density of the capsular material was the same from the inner to outer portions (Fig. 2a), but in ultrathin section specimens, the density of capsular materials in the outer portion was lower than that in the inner one (Fig. 2b). At higher magnification of the replica membrane, fine microfibrils were directly attached to the cell wall (Figs. 3a and 3c). In the ultrathin section of the brain lesion, a clear halo was observed between the capsule and the cell wall (Fig. 3d), while it was not observed in vitro (Fig. 3b). Phagocytized yeast cells were observed in vivo. The structure of the capsule was better preserved than that of the cell body (Fig. 4).

Cell wall. Thin layer of the cell wall was evident beneath the capsular microfibrils (Fig. 1). In the outer part of the cell wall, a particle-accumulating layer was observed (Fig. 1c). The diameters of particles were ranged from 15 to 25 nm. The particle-accumulating layer was thicker in vivo than in vitro (Figs. 3a and 3c). In the Ultrathin section, a transparent zone was observed in the middle of the cell wall in vivo (Fig. 3d), while it was absent in vitro (Fig. 3b).

Plasma membrane. The plasma membrane occasionally invaginated into the peripheral cytoplasm (Figs. 1, 2b, 3b, and 3d). These invaginations of the plasma membrane consisted of long and short fissured in the replica membrane (Fig. 1b).

Organelles. Various cellular organelles such as mitochondria (Figs. 1a and 2b), vacuoles (Fig. 2), lipid droplets (Figs. 1a and 2b), glycogens (Fig. 2) and a nucleus with prominent nuclear pores (Fig. 1a) were clearly observed by the QF-DE method.

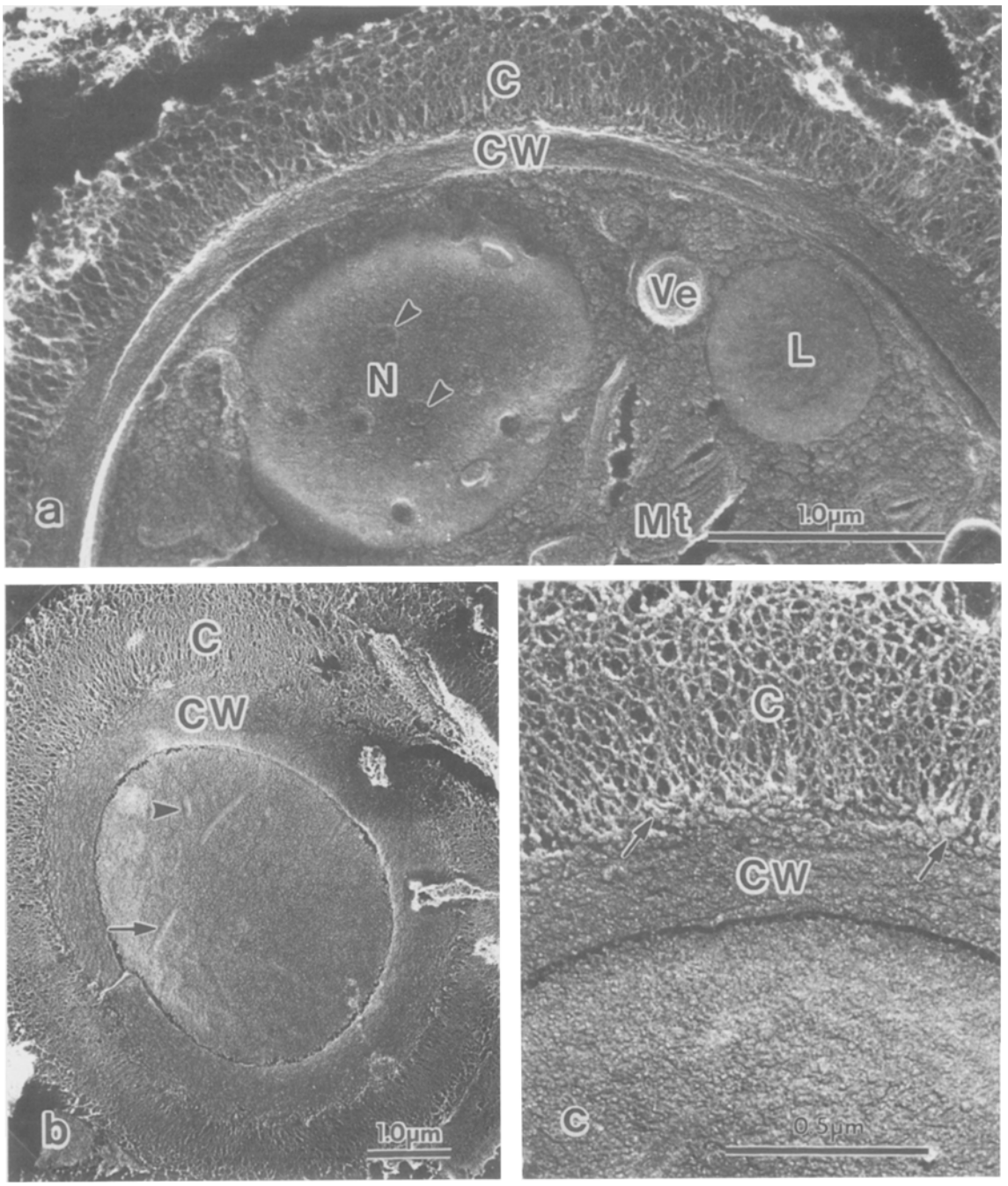

Fig. 1. QF-DE replica electron micrographs of unfixed in vitro cells of *C. neoformans*. A capsule (C) composed of fine microfibrils surround the cell wall (CW). (a) A nucleus (N), nuclear pores (arrowheads), mitochondria (Mt), vesicles (Ve) and lipid droplets (L) are observed in the cytoplasm. (b) On the surface of the cell membrane, long (arrow) and short (arrowhead) invaginations are observed. (c) The capsule is composed of fine microfibrils (10–13 nm in diameter), which branched off and are fused together, being directly attached to the cell wall. In the outer part of the cell wall, a particle-accumulating layer (arrow) is observed.

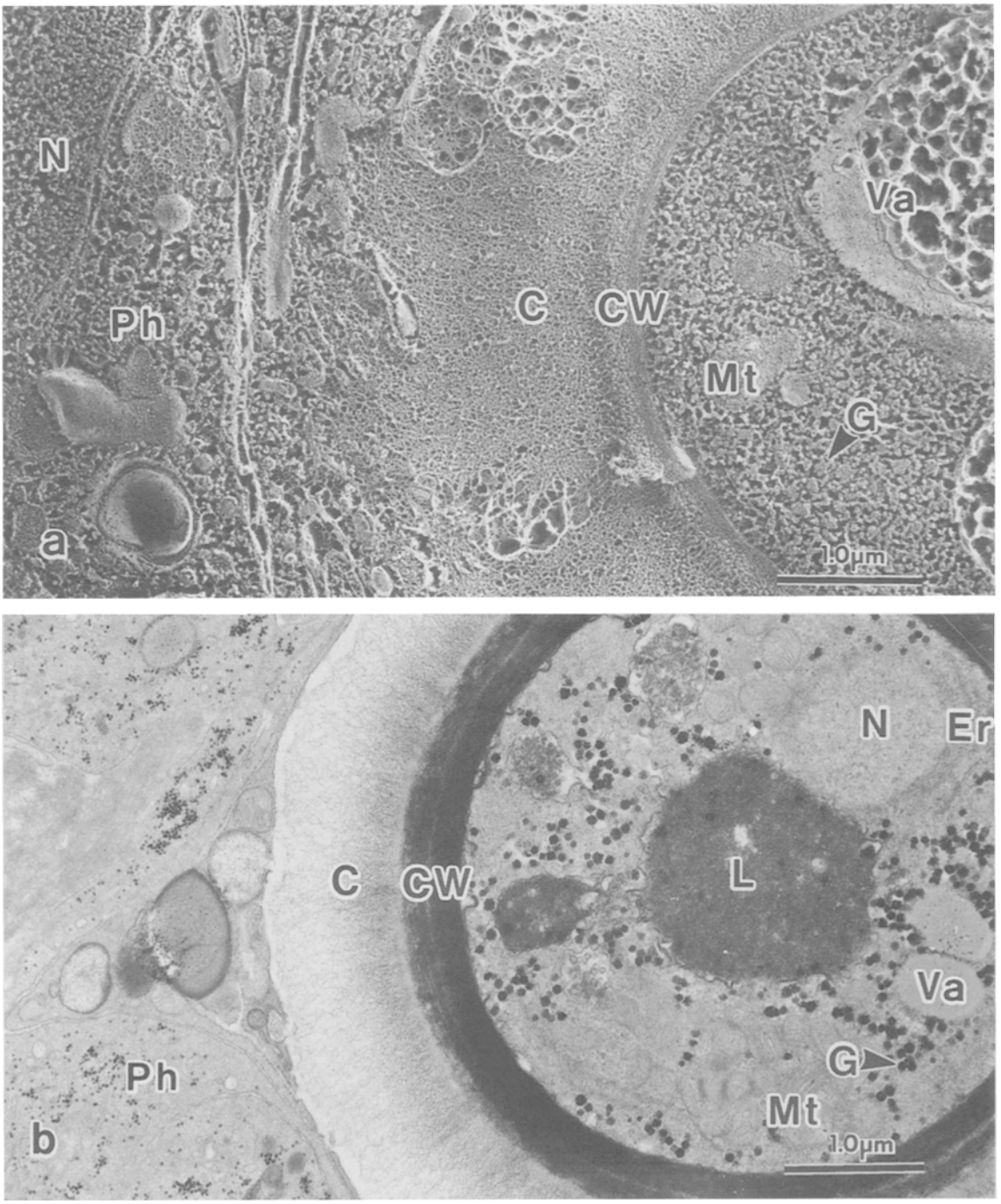

Fig. 2. Fixed in vivo cell. (a) A QF-DE replica electron micrograph of a freeze-fractured yeast cell surrounded by a phagocytic cell (Ph). The density of capsular microfibrils is nearly the same from the inner to outer portions of capsule (C). (b) An ultrathin section of an area corresponding to (a). The density of the outer portion of the capsule is lower than that of the inner. N: nucleus; CW: cell wall; Va: vacuole; L: lipid droplet; Er: endoplasmic reticulum; Mt: mitochondria; G: glycogen.

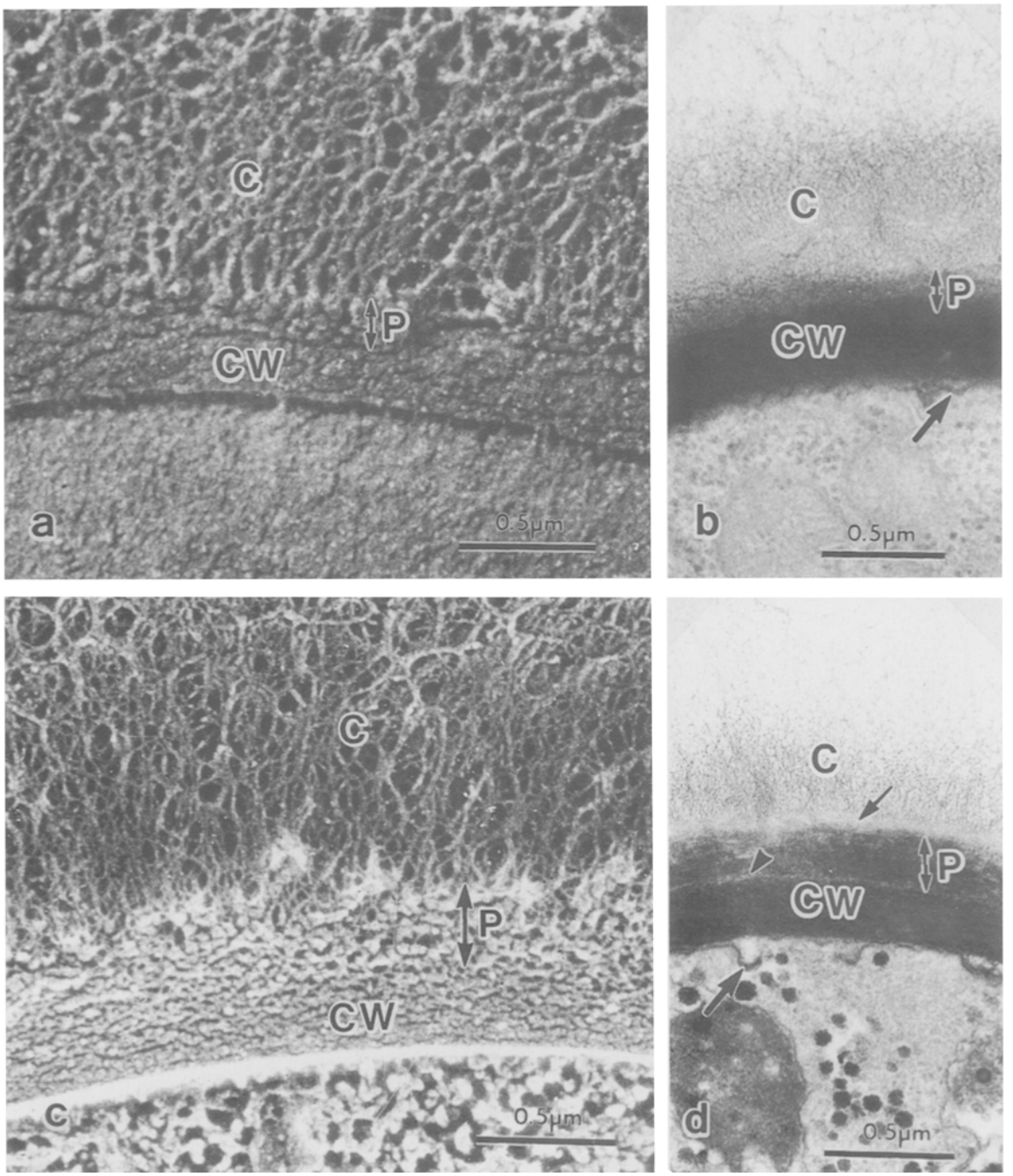

Fig. 3. Comparison of the capsule between in vitro (a, b) and in vivo (c, d) of fixed cells. (a) A QF-DE replica electron micrograph of the outer part of the cell wall (CW) is composed of a thin particle-accumulating layer (P). The particle-accumulating layer of an in vitro cell is thinner than that of an in vivo cell. (b) An ultrathin section of an in vitro cell, halos and transparent zones are not observed. (c) A QF-DE peplica electron micrograph of an in vivo cell shows a thicker particle-accumulating layer located at the outer part of the cell wall. (d) An ultrathin section of an in vivo cell, halos (small arrow) and transparent zones (arrowhead) are observed. Cell membrane often invaginates into the cytoplasm (large arrow). C: capsule.

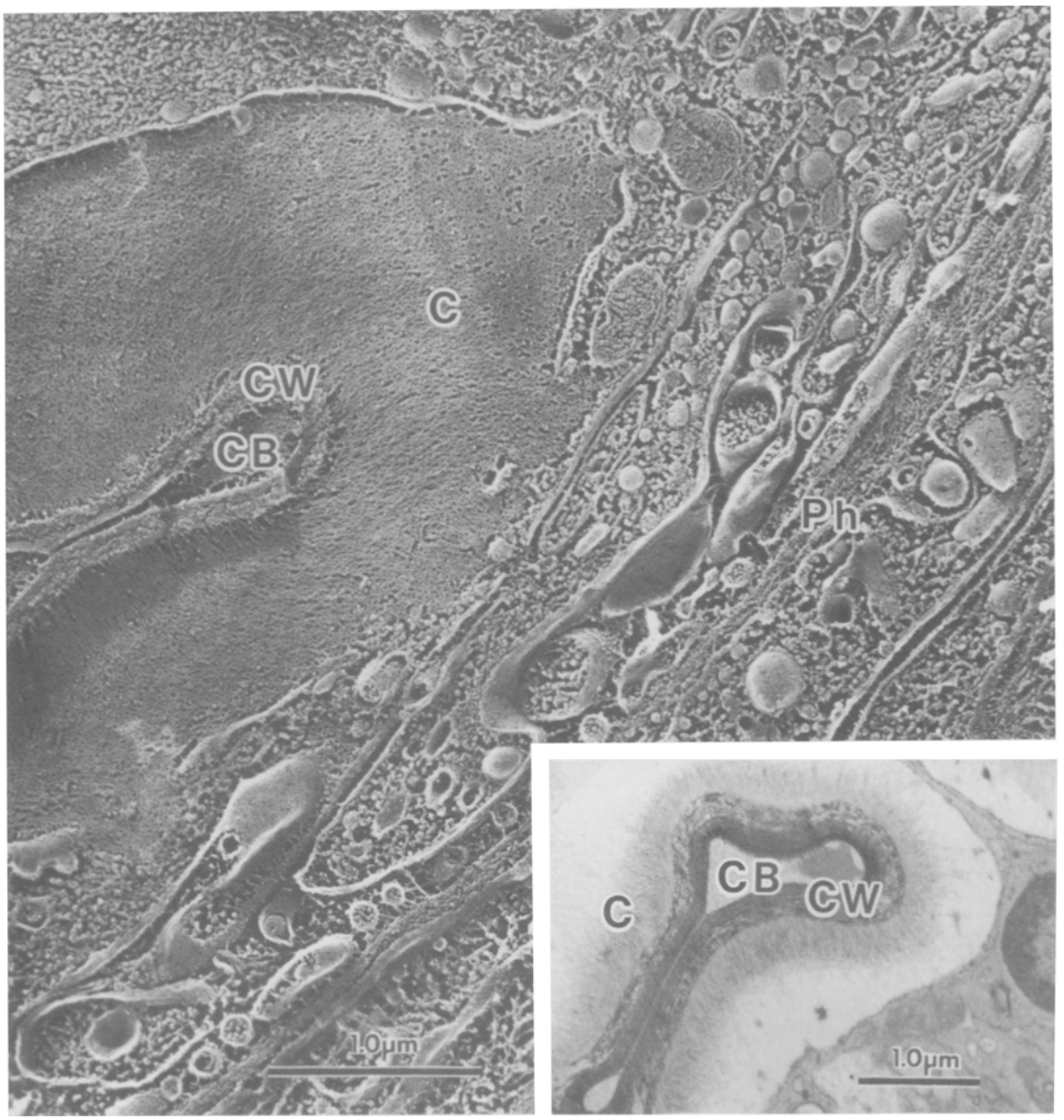

Fig. 4. A QE-DE replica electron micrograph of a fixed phagocytized yeast cell. Although the cell body (CB) is destroyed, the capsular structure (C) is well preserved. *Inset*: An ultrathin section of an area corresponding to A QF-DE replica. CW: cell wall; Ph: phagocyte.

Discussion

The QF-DE method clearly revealed a fibrillary structure in the capsule. The most interesting finding is the particle-accumulating layer between the cell wall and the capsule. Takeo et al. reported the existence of this layer comparing *C. neoformans* in vitro and in vivo, by conventional freeze-etching methods [15, 16]. They found that the cells contained more vesicles in the cytoplasm in vivo than in vitro and suggested that the precursors of the capsular material were synthesized

in these vesicles, secreted to outer layer of the cell wall, and polymerized to microfibrils of the capsule in the outer layer of the cell wall. Unfortunately, since their techniques could not reveal the granular structure clearly, the existence of precursor particles was uncertain. Our technique made it possible to clarify the existence of the precursor particle-accumulating layer. By comparing the thicknesses of the particle-accumulating layers between in vitro and in vivo, we found that the capsule was thicker in vivo than in vitro. This result suggests that the particle-accumulating layer consists of precursors or precursor-associated materials of the capsule.

In QF-DE replicas, capsular microfibrils branched off and fused together to form meshworks, as shown in Figs. 1 and 3. The microfibrils were directly attached to the cell wall. In the ultrathin section, the halo between the capsule and the cell wall and a transparent zone in the middle of the cell wall were observed in in vivo cells. This halo has been previously reported by many researchers [5–14]. The layer between this halo and the transparent zone seems to correspond to the particle-accumulating layer in the replicas. Although it has been reported to be a part of the cell wall [5–14], it should be categorized as a component of the capsule from its character. So we conclude that the halo and transparent zone may be artifacts caused by contraction of the particle-accumulating layer during fixation and embedding procedures. It is our opinion that the particle-accumulating layer tended to contract during fixation or embedding of its granular structure. Therefore, the ultrathin section of in vitro cells with a thin particle-accumulating layer does not show either a clear visible zone or a transparent zone, as shown in Fig. 3b.

In vivo (Figs. 2, and 4), the density of microfibrils in the capsule, observed in ultrathin sections, was different, being especially lower in the outer portion. However, the density of the capsule was the same from the inner to outer portions, as revealed in the replicas. These results suggest that a decrease of electron density of the capsule in the outer portion is due not only to physical extraction, but also to loss of electron density during fixation and washing. Meno & Amako [23] reported that the loss of density in the outer portion of the capsular material of *Klebsiella pneumonia* was due to the loss of electron density, and not to physical extraction. The same phenomenon may have occurred in experimental conditions of *C. neoformans*.

The cell body of phagocytized *C. neoformans* was severely destroyed, whereas the structure of its capsule was better preserved. This suggests that the capsular material is resistant to the digestion by lysosomal enzymes of phagocytic cells. Although further studies are necessary to clarify the pathogenic features of the capsule, its stability may be one of the important pathogenic factors of cystic lesions of *C. neoformans*.

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