SCRIPTA MEDICA (BRNO) – 73 (6): 369–380, *December 2000*

MORPHOLOGICAL TRANSITIONS DURING THE CELL DIVISION CYCLE OF *CRYPTOCOCCUS NEOFORMANS* AS REVEALED BY TRANSMISSION ELECTRON MICROSCOPY OF ULTRATHIN SECTIONS AND FREEZE-SUBSTITUTION

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A s t r a c t

Cryptococcus neoformans cells, glutaraldehyde fixed or freeze-substituted, were studied by transmission electron microscopy of ultrathin sections with the aim to characterise different morphological phases of the cell division cycle. Six stages were distinguished as follows: 1) A single mother cell with a filamentous capsule on the multilamellar cell wall and with typical membrane organelles (nucleus, mitochondria, vacuoles, endoplasmic reticulum and secretory vesicles). 2) The cell with a cylindrical protrusion resembling a sterigma developed on the mother cell. It was covered with a thin, electron-transparent wall and contained numerous secretory vesicles. 3) The cell with an isodiametrical bud differentiated from the cylindrical, sterigma-like protrusion. It comprised secretory vesicles, mitochondria and vacuoles and was covered with a thin, electron-transparent wall. 4) The cell with a nucleus that migrated into the bud and subsequently entered nuclear division. 5) The cell after nuclear division and before cytokinesis. 6) The cell at cytokinesis characterised by septum formation in the bud neck and separation of the daughter from the mother cell. Therefore, the cell division cycle in *C. neoformans* comprised three stages involved in interphase (mother cell, sterigma-like protrusion and bud development) and three stages of the M phase (migration of the nucleus followed by mitosis, state after mitosis and before cytokinesis, and cytokinesis). In visualising cell structures, freeze-substitution provided a better resolution of intracellular components, while the outer structures (cell wall and capsule) were better discernible after glutaraldehyde fixation.

K e y w o r d s

Cryptococcus neoformans, cell division cycle, transmission electron microscopy, ultrathin sections, freeze-substitution

INTRODUCTION

Cryptococcus neoformans, the haploid phase *of Filobasidiella neoformans (1)*, is an important human fungal pathogen *(2)* belonging to the basidiomycetous yeasts (*3, 4).* It causes cryptococcosis, a life-threatening condition in immunodeficient and

immunosupressed patients *(5, 6, 1)*. Since common anti-fungal agents are potentially toxic or have limited efficacy *(7, 6*), new ways of effective therapy for cryptococcosis have been searched and, among other approaches, attention has been focused on the cell wall as a target of therapeutic intervention and on the cytoskeleton because a wide range of its inhibitors is available. However, this therapy can be successful only if the organisation and functions of the cytoskeleton in this pathogen are well understood. In spite of the existence of a large number of studies on *C. neoformans,* the cytoskeleton has not attracted attention, with the exception of microtubules studied at the ultrastructural level *(9, 10).*

We have recently published a detailed description of the actin cytoskeleton and microtubules in *C. neoformans* during cell budding *(11)*. This paper is a complement to our previous study and provides a description of morphological transitions during the cell division cycle studied at the ultrastructural level in ultrathin sections.

MATERIALS AND METHODS

Yeast strains

Human pathogenic strains of *Cryptococcus neoformans,* designated IFM 45842, IFM 40042, IFM 41464 and IFM 45941, from the Culture Collection of the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Japan *(12)* were used.

Media and cell cultivation

Cells were cultivated in YPG medium (1% (w/v) yeast extract, 1% (w/v) pepton and 1% (w/v) glucose *(13))* or in YEPD medium (1% (w/v) yeast extract, 2% (w/v) bactopepton and 2% (w/v) glucose, *(14))* on a shaker overnight at 30 °C.

Fixation for fluorescence microscopy

Cells of exponential cultures were fixed using 5% (w/v) paraformaldehyde in phosphate buffer, pH 6.9, containing 1.25 mM EGTA and 1.25 mM $MgCl_2$, for 10 or 90 minutes and were washed three times with buffer without the fixative.

Calcofluor white staining

Calcofluor white, 0.5% (w/v) concentration, was added to growing cells to give a concentration of 0.005% and the culture was incubated for 15 min. The stained cells were immediately fixed and washed as described above.

Nuclear staining

Nuclei were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) dissolved in the Vectashield mounting medium (Vector Laboratories) to a final concentration of 1 $\mu g\ m^{l-}$.

Phase-contrast and fluorescence microscopy

Olympus BH2-RFCA, Leica Laborlux S Leitz, and Jenalumar fluorescence microscopes were used with standard filter blocks for violet (355–425 nm), blue (450–490 nm) and green (515–560 nm) excitation light, with a Plan Phaco 3 100/1.25 objective and with equipment for phase-contrast microscopy.

Ultrathin sections

Cells were fixed with 3% glutaraldehyde in 130 mM cacodylate buffer, pH 7.4, for 2 h, postfixed with 1% osmium tetroxide in the same buffer for 1 h, dehydrated in an alcohol series for 2 h, and embedded in the LR White resin and allowed to polymerise for 2 days at 60 °C. Ultrathin sections were contrasted with 2.5% uranyl acetate for 30 min and with lead citrate for 6 min. The sections were viewed and photographed in a Tesla BS 500 transmission electron microscope at 90 kV.

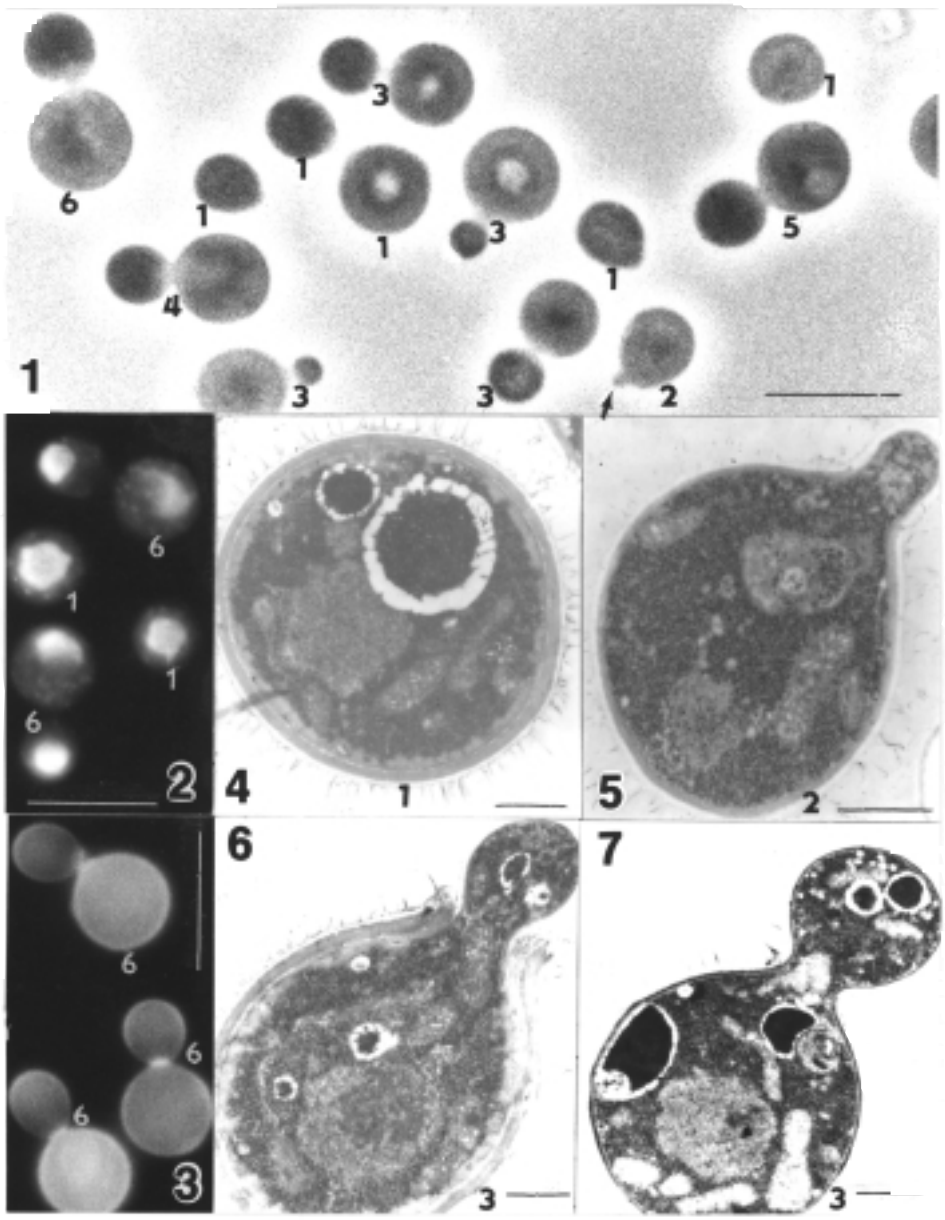
Freeze-substitution

Cells were collected by centrifugation and sandwiched between two copper disks. They were cryo-fixed by plunging into propane slush kept in liquid nitrogen. The specimens were freeze-substituted in 2% osmium tetroxide/aceton at –80 °C for 2 days and embedded in epoxy resin *(15)*. Ultrathin sections were stained with uranyl acetate and lead citrate and observed and photographed with a JEM-1200EX electron microscope (JEOL, Tokyo).

RESULTS

Phase-contrast and fluorescent microscopy of *Cryptococcus neoformans* cells

A culture in the exponential phase of growth viewed by phase-contrast microscopy showed spherical cells in different stages of reproduction by budding (*Fig.1).* Nuclei stained with DNA-probe DAPI were visualised by fluorescence microscopy *(Fig. 2).* Isolated mother cells, each containing one nucleus, were at the first stage of the reproduction cycle (*Figs 1,2)*. Cells that bore a cylindrical, sterigma-like protrusion were at the second stage *(Fig.1; arrow)* and cells which had an isodiametrically growing bud were at the third stage of the cell reproduction cycle. In budding cells, the nucleus migrated into the bud and, subsequently, mitotic nuclear division was initiated; this was followed by elongation of the nucleus that was then shared between bud and mother *(Fig. 1)*. These cells were at the fourth stage of the reproduction cycle. Cells after nuclear division *(Fig. 2)* but before cytokinesis (septum formation) were at the fifth stage *(Fig. 1).* Cells forming a septum and subsequently separating were at the sixth stage of the reproduction cycle, as demonstrated by Calcofluor white staining of the wall and septum *(Fig. 3).* There was a difference in the intensity of fluorescence between the daughter and the mother cell *(Fig. 3).*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Ultrastructure of *Cryptococcus neoformans* revealed by transmission electron microscopy in ultrathin sections of chemically-fixed cells

The six different morphological stages of reproduction by budding were also observed by transmission electron microscopy in ultrathin sections through fixed cells. Stage 1 of the cell division cycle, i.e., isolated mother cells, was characterised by the presence of a single nucleus in each cell, by several mitochondria, vacuoles, endoplasmic reticulum and numerous ribosomes. There was a cortical layer composed of a finely granular material of unknown nature. The lamellar cell wall and filaments of capsule radiating in all directions were the characteristic features of the cell surface (*Fig.* 4).

Cells with short, cylindrical, sterigma-like protrusions were typical of stage 2 *(Fig.5)*. This cylindrical structure arose as the first stage in the development of a new daughter cell and resembled the cylindrical cell of fission yeasts. The sterigma-like protrusion contained numerous secretory vesicles and ribosomes and had a thin, electron-lucid cell wall on its surface.

The differentiation of an isodiametrical bud from the cylindrical sterigma-like protrusion was stage 3 *(Fig. 6,7,11).* This was characterised by isodiametric growth and multiple secretory vesicles, ribosomes, mitochondria and vacuoles. Inside the bud, there was evidence of further cell structures being delivered from the mother cell to the bud.

Fig. 1

Exponential culture of *C. neoformans*. Cells are designated by numbers corresponding to the morphological stages of the cell division cycle. Phase-contrast microscopy. Magnification, x 1800. Bar 10 μm.

Fig. 2

Cells of an exponential culture stained with DNA-probe DAPI for nuclei. Cells only in stage 1 and 6 are shown. Magnification, x 1700. Bar, 10 μm.

Fig. 3

Cells of an exponential culture, stained with Calcofluor white to detect the wall and septum. Magnification, x 1800. Bar 10 μm.

Fig. 4

Transmission electron micrograph (TEM) of glutaraldehyde fixed *C. neoformans* cells (stage 1).Magnification, x 9200. Bar 1 μm.

Fig. 5

TEM of an ultrathin section through a glutaraldehyde-fixed *C. neoformans* cell. In the cytoplasm of a “sterigma” (stage 2), ribosomes and secretory vesicles can be seen. Magnification, x 12000. Bar 1 μm.

Figs 6 and 7

TEMs of two different glutaraldehyde-fixed budding cells of *C. neoformans* (stage 3). Nucleus, mitochondria, vacuoles, secretory vesicles are visible. Magnification x 9000. Bar 1 μm.

Migration of the nucleus from the mother cell to the bud was a characteristic event for stage 4 *(Fig. 8).* The mother nucleus produced a finger-like protrusion and extended it deeply into the bud. At this stage, massive transport of mitochondria, endoplasmic reticulum and fine cortical material of unknown nature to the bud was also detected. After migration into the bud, the nucleus began to divide and subsequently elongated, extending into the mother cell. The elongation was followed by nuclear segregation leading to that one nucleus appeared in the bud and the other in the mother cell.

A short period between mitosis and cytokinesis represented stage 5 *(Fig. 9).* Cell organelles were partitioned and distributed between the mother and daughter cells resulting in that both cells possessed all cell structures (one nucleus with a nucleolus, mitochondria, vacuoles, endoplasmic reticulum, ribosomes and secretory vesicles); however, cytokinesis had not started yet. Of interest was the finding of mitochondria that accumulated at the bud neck before cytokinesis was initiated *(Fig. 9).*

Cytokinesis was the last stage of reproduction by budding *(Fig. 10).* The plasma membrane produced an invagination at the bud neck (not shown) that led to septum formation *(Fig. 10).* Stage 6 was completed by cell separation, giving rise to a mother and a daughter cell both being at the first stage of the subsequent cell division cycle.

Comparison of the ultrastructure of *C. neoformans* cells observed in ultrathin sections after chemical fixation and freeze-substitution

Freeze-substituted cells of *C. neoformans* showed inner cell structures at all stages of the cell division cycle at a high resolution. Especially, membrane structures and ribosomes were disclosed very clearly *(Fig. 12),* when compared with glutaraldehyde-fixed cells observed at similar magnifications *(Fig. 11).* With the use of freeze-substitution, the plasma membrane and the membranes of other cell structures could be clearly distinguished only when sectioned perpendicularly. The wall of the daughter cell consisted of a thin layer, while the mother cell wall was thicker and composed of several layers. Electron-transparent vesicles seen close to the plasma membrane *(Fig. 12)* were similar to those observed in the cells treated by chemical fixation *(Fig. 11).* On the other hand, the multilamellar wall and capsule were visualised more clearly in chemically-fixed cells *(Figs. 4,6, 8)* than in freeze-substituted cells *(Fig.12),* particularly if the cells were old.

DISCUSSION

Morphological transitions in the cell division cycle of *Cryptococcus neoformans*

Transmission electron microscopy of glutaraldehyde-fixed cells showed six morphologically distinct stages in the cell division cycle of *C. neoformans.* Three

stages took place during interphase and three during M-phase. Stages 1,2 and 3 were characterised by the development of a new daughter cell on the mother cell, while stages 4, 5 and 6 included nuclear division and cytokinesis followed by cell separation (*Fig. 13*).

The presence of a multilamellar wall revealed after glutaraldehyde fixation was an interesting finding. It was seen only in some of the mother cells from the exponential culture; it has previously been described after fixation with potassium permanganate or other fixation procedures, including acrolein *(16, 17)*. While the multilamellar wall was observed only on some mother cells, the growing daughter cell always had a thin electron-lucid wall. It is probable that this difference in cell wall structure is related to the age of cells and that the mother wall is either restructured or completed with a new material. Our recent findings of actin patches randomly surrounding the cell cortex in *C. neoformans* cells *(11)* may be associated with the process of wall reconstruction.

The inner ultrastructure of *C. neoformans* cells at this stage fully corresponded to that presented previously by *in vivo* freeze-fracture techniques *(18).*

The formation of a thin cylindrical protrusion, similar to the sterigma in *Fellomyces fuzhounesis,* at the first stage of daughter cell development *(19)* was another interesting finding. In *F. fuzhounesis,* sterigma formation is the first stage of conidiogenesis *(20, 19)*, in *C. neoformans,* the cylindrical protrusion is part of the budding process. Both these organisms have been included in the *Tremellales* clade on the basis of their taxonomy *(3, 4)* and this is supported by the fact that the cylindrical protrusion which, in *Cryptococcus,* is short and differentiates into a bud, in *Fellomyces,* presents as a sterigma, a long protrusion differentiating into a conidium by a bud-like process. This also demonstrates the great morphological diversity so typical of fungi.

The development of a sterigma-like protrusion may be related to the microtubular skeleton recently identified in sterigmata of *C. neoformans (11)*. Microtubules may provide a pathway for transport of secretory vesicles to the growing apex.

The development of an isodiametrical bud at the third stage of interphase was associated with the presence of secretory vesicles, mitochondria and vacuoles, which suggests intensive growth or metabolism of the cell. This stage was also characterised by a network of microtubules that might provide tracks for secretory vesicles involved in the isodiametrical bud growth. The subcortical distribution of actin patches (*11*) and the presence of ribosomes were also probably related to the isodiametrical growth as were mitochondria, a source of energy for the bud.

Stage 4 of the cell reproduction cycle in *C. neoformans* included migration of the nucleus into the bud where nuclear division was initiated. This is a typical feature of the basidiomycetous yeasts including *C. neoformans (21, 22, 23, 9, 10, 24)*. These processes have been described with the use of transmission electron

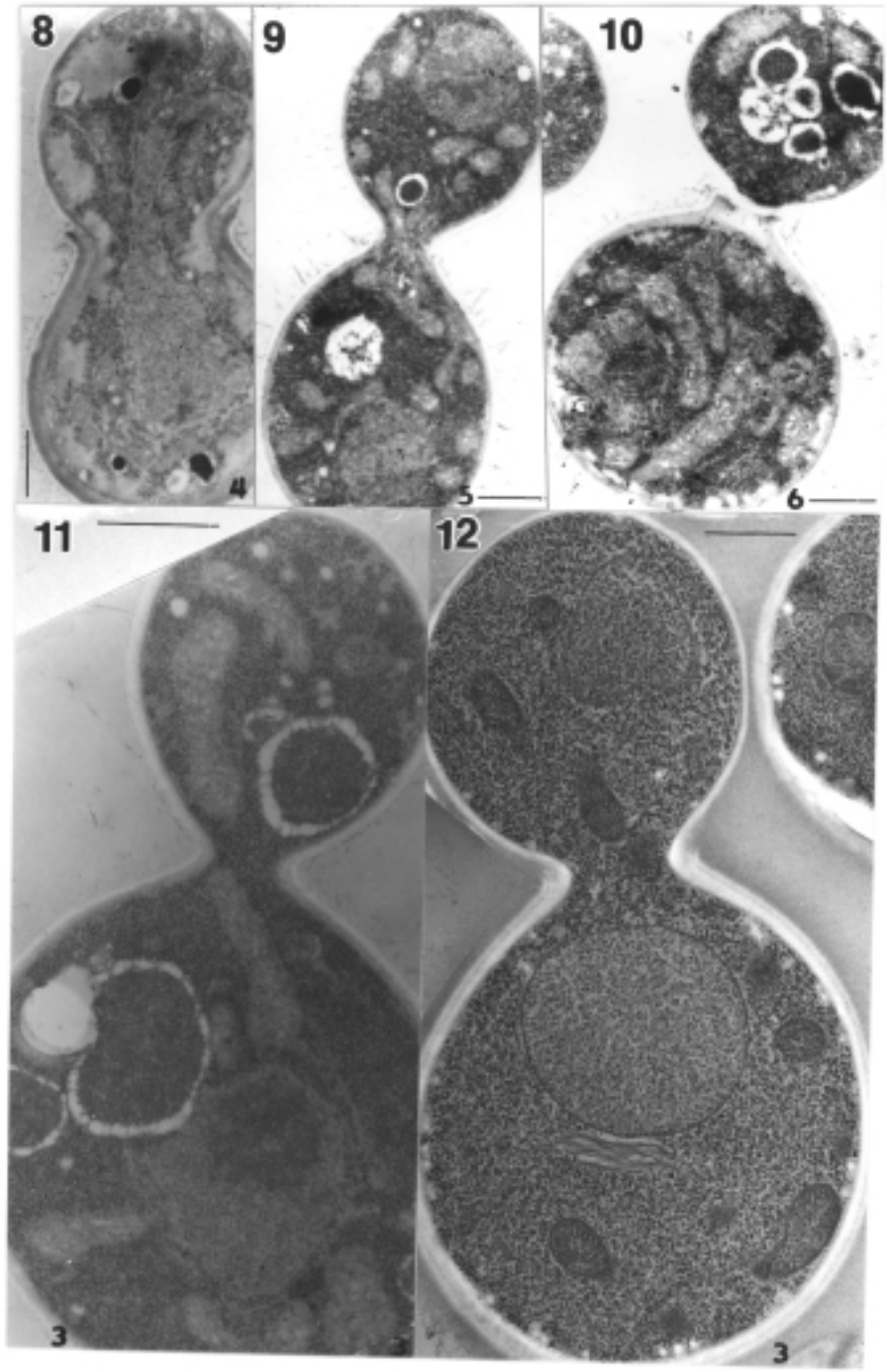
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Fig. 8
TEM of a glutaraldehyde-fixed budding cell of *C. neoformans* (stage 4). Nucleus migrating into the bud is visible. Magnification, x 9000. Bar 1 μm.

Fig. 9
TEM of a glutaraldehyde-fixed mother-daughter cell of *C. neoformans* after mitosis but before cytokinesis (stage 5) containing two nuclei and two sets of complete cell structures (mitochondria, vacuoles, secretory vesicles and endoplasmic reticulum). Magnification, x 9000. Bar 1 μm.

Fig. 10
TEM of glutaraldehyde-fixed cells of *C. neoformans* at the stage of cytokinesis (stage 6) with a fully developed septum in the bud neck. Magnification, x 9000. Bar 1μm.

Fig. 11
TEM of a glutaraldehyde-fixed budding cell of *C. neoformans* (stage 3). Nucleus, mitochondria, vacuoles, secretory vesicles, endoplasmic reticulum and ribosomes are visible. Magnification, x 17000. Bar, 1 μm.

Fig. 12
TEM of a freeze-substituted cell of *C. neoformans* at stage 3. Membrane structures and ribosomes are clearly disclosed. Magnification, x 13000. Bar 1 μm.

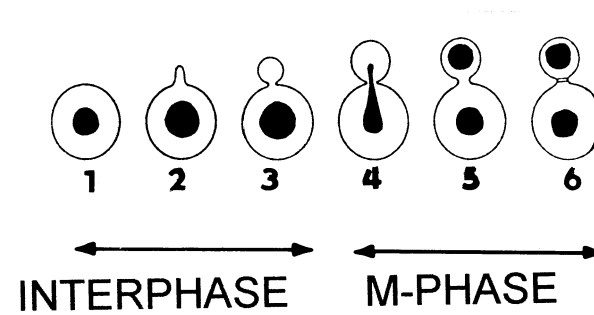

Fig. 13
Schematic drawing of six morphologically distinct stages in the cell division cycle by budding in *C. neoformans.* Stages 1, 2 and 3 take place during interphase, stages 4, 5 and 6 are included in the M phase.

microscopy *(9)* and recently confirmed by a computer-aided, three-dimensional reconstruction of nuclear migration and division *(10).* In addition, partial breakdown of the mitotic nuclear envelope reported in some *Basidiomycetes (21, 22, 23, 25, 26)* is also one of the characteristic features of *C. neoformans (9, 10)*.

The development of an actin ring *(11),* a characteristic feature of stage 5, could not be clearly identified at the ultrastructural level. At stage 6, the morhology of septum formation corresponded to that of the *Basidiomycetes*, as already reported (*16,17*).

It remains to be determined what is the role of microtubules and actin in the different stages of the cell reproduction cycle reported in this work. The use of actin and microtubular inhibitors seems to be the most convenient approach for our future investigation.

Time-course of the cell cycle stages in *Cryptococcus neoformans*

Takeo et al. *(13)* studied DNA content in cells of a stationary *C. neoformans* culture by quantitative fluorescence microscopy after DNA staining with propidium iodide or DAPI and found that, in unbudded cells, DNA content corresponded to that in either G2 (replicated nuclear DNA) or G1 (non-replicated nuclear DNA). On the basis of these results it can be speculated that, in a single, isodiametrically growing *C. neoformans* cell, both G1 and S phases may take place without any daughter cell development. Consequently, the development of both the cylindrical protrusion and the bud may be triggered only after the cell has passed through the checkpoint in the G2 phase. If our interpretation is correct, stage 1 in our experiments may include both G1 and S phases, while stages 2 and 3 take place within the G2-phase of the cell division cycle.

Generally, the M-phase involves nuclear migration, mitotic nuclear division and cytokinesis. These were identified in our experiments as morphologically distinct stages 4, 5 and 6. However, our classification of the nuclear migration stage is still questionable. *Mochizuki et al. (9)* designates the nuclear protrusion in the bud as “prophase”. In contrast, our previous study showed that cytoplasmic microtubules disappeared only after the whole nucleus was inside the bud *(11)*. To unify the interpretation, we took into consideration both our and Mochizuki’s findings and regarded stage 4 as a period including nuclear migration and nuclear division. This was also the first stage of the M-phase, which was followed by segregation and migration of the nuclear material into each of two daughter cells (stage 5) and by cytokinesis (stage 6).

In conclusion, the cell division cycle in *C. neoformans* can be defined as a sequence of six morphologically distinct steps, of which the first three are included in interphase and the other three take place in the M phase.

ACKNOWLEDGEMENT

This study was supported by the Japanese Ministry of Education, Science and Culture that provided funding for Marie Kopecká, Guest Professor at Chiba University, Research Center for Pathogenic Fungi and Microbial Toxicoses, Japan, in 1998–1999 and by grant no. 310/00/0391 from the Grant Agency of the Czech Republic.

The authors thank to V. Ramíková, D. Klemová, P. Hnilička, H. Hromadová of the Department of Biology, Faculty of Medicine in Brno, for their technical assistance and to Dr. Z. Doležalová for kind reading the manuscript.

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MORFOLOGICKÁ STÁDIA V BUNEČNÉM CYKLU *CRYPTOCOCCUS NEOFORMANS* STUDOVANÁ TRANSMISNÍ ELEKTRONOVOU MIKROSKOPIÍ ULTRATENKÝCH ŘEZŮ A MRAZOVOU SUBSTITUCÍ

S o u h r n

Cryptococcus neoformans byl studován transmisní elektronovou mikroskopií ultratenkých řezů po glutaraldehydové fixaci a mrazové substituci s cílem charakterizovat morfologicky odlišné fáze v průběhu buněčného cyklu. Bylo zjištěno šest morfologicky odlišných stádií. 1) Samotná mateřská buňka s vláknitým pouzdrem na vícevrstevné buněčné stěně, obsahující při použitém zvětšení typické membránové organely jako jádro, mitochondrie, vakuoly, endoplasmatické retikulum, sekreční měchýřky, a nepravidelnou kortikální vrstvu. 2) Vznik cylindrického, sterigmatu podobného výběžku na mateřské buňce. Tento výběžek krytý tenkou elektron-transparentní stěnou obsahoval četné sekreční měchýřky. 3) Diferenciace isodiametrického pupenu z cylindrického výběžku; pupen, krytý tenkou elektron-transparentní stěnou, obsahoval sekreční měchýřky, mitochondrie a vakuoly. 4) Migrace jádra do pupenu, následovaná jaderným dělením, vyznačovala nástup M fáze buněčného cyklu. 5) Buňky po jaderném dělení, ale před cytokinesí. 6) Cytokinese, charakterizovaná vývojem septa v krčku pupenu, byla následována separací buněk. Cyklus buněčného dělení u *C. neoformans* sestává ze tří stádií inferfáze (mateřská buňka, tvorba "sterigmatu" a tvorba pupenu) a tří stádií M – fáze (jaderná migrace a mitosa, stav po mitose, ale před cytokinesí, a vlastní cytokinese). Mrazová substituce poskytla vyšší resoluci vnitrobuněčných strukur, zatím co vnější struktury (stěna a pouzdro) byly lépe rozlišitelné po glutaraldehydové fixaci.

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