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Original Article

Cytological Study of Cell Cycle of the Pathogenic Yeast *Cryptococcus neoformans*

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Abstract

Cryptococcus neoformans is an opportunistic human pathogen belonging to basidiomycetous fungi and has unique properties in cell cycle progression. The purpose of this study was to measure the duration of the cell cycle in this yeast. Under standard liquid culture conditions (1% yeast extract, 1% polypeptone, and 1% glucose; 24°C; and 150 rpm), the doubling time of exponentially growing *C. neoformans* was 132 ± 16 min (mean ± standard deviation), and the durations of the G1, S, G2, and M phases were about 71, 18, 25, and 18 min, respectively. DNA synthesis started before bud emergence, and finished by the time the size of the bud became 1/4 that of the mother cell. The doubling time of the daughter cells was about twice that of the mother cells. The spindle pole body was located on the outer nuclear envelope and showed a duplicated form from the G1 phase to the G2 phase. These data form a basis for further cell cycle study of *C. neoformans*.

Key words: cell cycle, *Cryptococcus neoformans*, DNA measurement, spindle pole body, electron microscopy

Introduction

Cryptococcus neoformans is the asexual stage of *Filobasidiella neoformans*. It belongs to the class Basidiomycetes and is distributed worldwide. It is an opportunistic human pathogen causing cryptococcosis and is one of the most life-threatening microorganisms in HIV-infected patients[1].

To manage cryptococcosis, it is essential to understand the biological properties of *C. neoformans*. This microbe has unique cell cycle features. Its growth arrests at not only the G1 phase but also the G2 phase in the stationary phase[2]. Bud emergence is gradually delayed from the S phase to the G2 phase with growth progression[3].

The purpose of this study was to measure the durations of the G1, S, G2 and M phases and the doubling times of the mother and daughter cells under standard liquid culture conditions. Electron microscopy of spindle pole bodies (SPBs) was also carried out since these organelles play an important role in cell cycle progression. The basic data obtained in this study should be useful for further studies of the cell cycle of *C. neoformans*.

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Materials and Methods

Strains, culture conditions, and measurements of doubling time in liquid culture

The strains used in this study were *C. neoformans* IFM 5808, 5844, 41464, and 45941[4]. They were haploid and used for a previous study[2]. Cells were cultured in 20 m*l* of YPG liquid medium (1% yeast extract, 1% polypeptone, and 1% glucose) in a 100-m*l* Erlenmeyer flask at 24°C with reciprocal shaking at 150 rpm[5]. They grew exponentially and reached stationary phase at 22-26 hr. The optical density at 660 nm (OD_{660}) of the culture was measured using a UV-1200 spectrometer (Shimadzu, Kyoto, Japan). The exponential phase cells used in the present analysis were 16-17 hr cultures whose

OD_{660} showed 1.0 to 4.0. The culture reaching stationary phase showed OD_{660} 25-30 under our culture conditions. The doubling times of exponential phase cells in liquid culture were determined by the time required to double OD_{660}.

DNA measurement of cells

The cells were fixed with 70% ethanol, washed with water, and suspended in NS buffer (10 mM Tris (pH 7.4), 0.25 M sucrose, 7 mM β-mercaptoethanol, 0.4 mM phenylmethylsulfonyl fluoride, 1 mM disodium EDTA, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 0.1 mM $CaCl_2$). They were stained with propidium iodide (5 μg/m*l*) containing ribonuclease A (0.5 mg/m*l*, Sigma R-5503) at 37°C for 2-4 hours. They were then examined with an Olympus BHS-RFC microscope equipped with Olympus OSP-1, and fluorescence intensity was recorded as the DNA amount for individual cells of specific cell cycles[6].

DNA content was also determined with a laser scanning cytometer (LSC model 101, Olympus, Tokyo, Japan) for several to ten thousands of cells. These were analyzed manually to determine duration time of G1 phase, as well as S, G2 and M phases[3].

Time-lapse microphotography and doubling time measurements in petri-dish culture

The cell division of exponentially growing *C. neoformans* in YPG medium at 24°C was monitored and photographed at 10-15 min intervals for 6-9 hr using a Nikon inverted microscope in petri-dish culture. Ten μ*l* of cell suspension in the exponential phase of liquid culture was inoculated into 0.5 m*l* of YPG medium in a plastic petri-dish coated with poly-D-lysine to prevent cell movement during the observation. The daughter cells were separated immediately after cytokinesis, and the time of cell separation was regarded as the time of cell division. The doubling times of mother and daughter cells were recorded individually as times required to make cell divisions, whereas population doubling time of cells in petri-dish culture was measured by the time required to double the cell number (e.g., from 50 to 100).

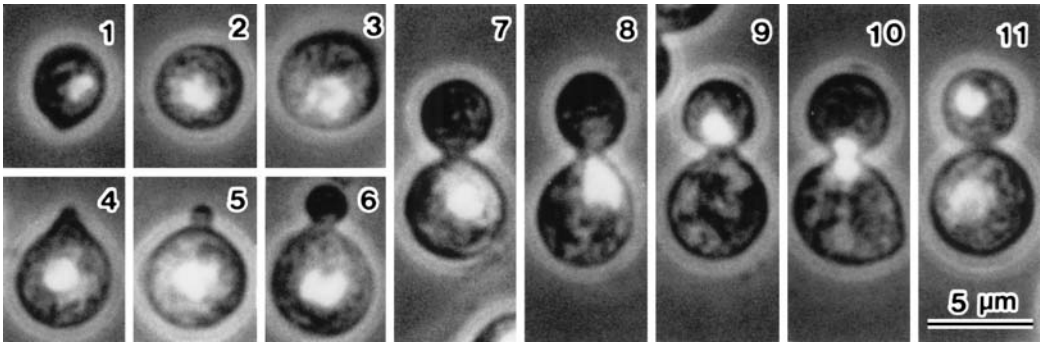
Electron microscopy

The cells were collected by centrifugation, sandwiched between two copper discs, and rapidly frozen using propane slush cooled in liquid nitrogen. They were freeze-substituted in acetone containing 2% osmium tetroxide at –80°C for 2-3 days and embedded in epoxy resin[7, 8]. Ultrathin sections were obtained using a diamond knife, stained with uranyl acetate and lead citrate[9], and examined by a JEM 1200EX transmission electron microscope (JEOL Ltd., Tokyo).

Results

Stages of cells that reflect cell cycle progression and their proportion

Cells in the exponentially growing phase were stained for DNA, and examined by phase-contrast and fluorescence microscopes. They



Stage	1	2	3	4	5	6	7	8	9	10	11
Proportion (%)	←	48.7 ±5.0	→	7.6 ±1.8	5.2 ±2.7	4.8 ±0.9	8.7 ±2.8	5.1 ±0.6	4.9 ±0.3	3.5 ±1.1	11.5 ±1.4

Fig. 1. Stages of *Cryptococcus neoformans* cells in exponential phase and proportions of each stage. Cells were classified into 11 stages by size, bud size, and nuclear position. Proportions (%) are shown as means ± standard deviations. Data were collected from 4 independent experiments using IFM 45941 and 5844 strains. More than 200 cells were counted in each experiment.

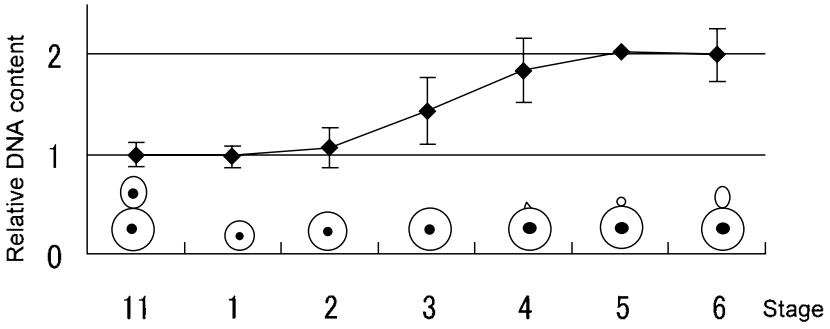


Fig. 2. DNA measurements in individual *C. neoformans* IFM 5844 cells. DNA synthesis began in medium-sized unbudded cells (stage 2) and finished in cells with small buds (stage 5). One (stage 5) to 27 (stage 2) cells were measured in each stage, and values are shown with standard deviations (except stage 5).

were classified into 11 stages by cell size, bud size, and nuclear position as follows (Fig. 1): 1) small unbudded cells, corresponding to newborn daughter cells; 2) medium unbudded cells, corresponding to growing daughter cells; 3) large unbudded cells, corresponding to full-grown daughter or mother cells; 4) cells with pimple-like buds; 5) cells with small buds (length of bud: less than 1/4 that of the mother cells); 6) cells with medium-sized buds (length of bud: 1/4 to 1/2 that of the mother cells); 7) cells with large buds (length of bud: greater than 1/2 that of the mother cells); 8) mitotic cells with nuclei in mother cells; 9) mitotic cells with nuclei in daughter cells; 10) mitotic cells with nuclei between daughter and mother cells; and 11) cells that had finished cytokinesis but had daughter cells still attached to the mother cells.

Cells in each stage were enumerated and calculated for frequency (Fig. 1).

DNA amount in each stage of cell cycle

By measuring relative DNA content in individual cells using propidium iodide staining, it was found that DNA synthesis started before bud emergence (stage 2) and finished at the stage when the cells had small buds (stage 5) (Fig. 2).

Analysis by laser scanning cytometry

In order to determine duration time of the G1 phase in exponential phase cells in liquid culture, it was necessary to determine the proportion of G1 phase cells by analyzing the proportion of cells that had not started DNA synthesis. Figure 3 shows the cell distribution of various DNA amounts determined by laser scanning cytometry. The first peak of smaller DNA amount corresponds to cells in the G1 phase, which occupied 54.0% of the total cell. The second peak of larger DNA amount and cell populations between the two peaks corresponds to cells in the S, G2, and M phases.

Duration of cell cycle in exponentially growing *C. neoformans* in liquid culture

Cells in stages 5-7 were in the G2 phase, since they had completed DNA synthesis but had not started mitosis (Figs. 1 and 2). They accounted for about 18.7% (5.2% + 4.8% + 8.7%) of the total cell population (Fig. 1). Cells in stages 8-10 were in the mitotic phase, judging by nuclear position, and were about 13.5% (5.1% + 4.9% + 3.5%) of the total cell population (Fig. 1). Duration of S phase can be calculated by subtracting duration times of G1, G2, and M phase from the entire cell cycle duration (100% – 54.0% – 18.7% – 13.5% = 13.8%). Thus, duration of the G1, S, G2, and M phases in exponentially growing *C. neoformans* would be 54.0%, 13.8%, 18.7%, and 13.5% per cell cycle, respectively (Fig. 4). Because the doubling time of the exponentially growing cells under

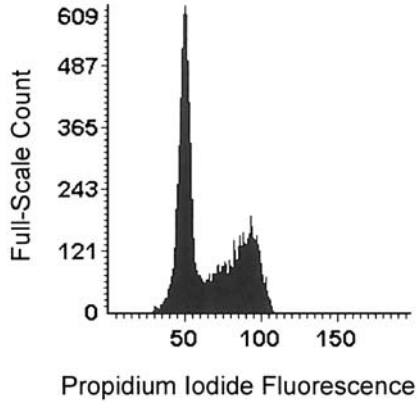


Fig. 3. Analysis of cell population of *C. neoformans* IFM 5844 cells by laser scanning cytometry. The first peak corresponds to G1-phase cells, which were found to make up about 54 % of the population. Similar results were obtained in 4 other experiments.

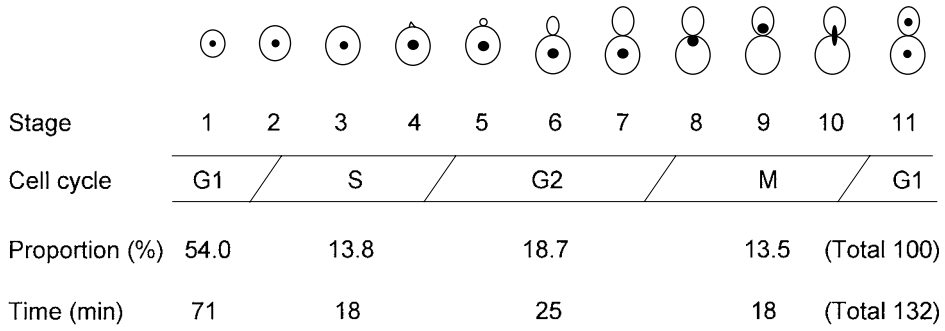


Fig. 4. Duration of cell cycle of exponentially growing *C. neoformans* in liquid culture. The durations of the G1, S, G2, and M phases were estimated from the frequency of each stage (Fig. 1), DNA measurements (Fig. 2), and analysis by laser scanning cytometry (Fig. 3).

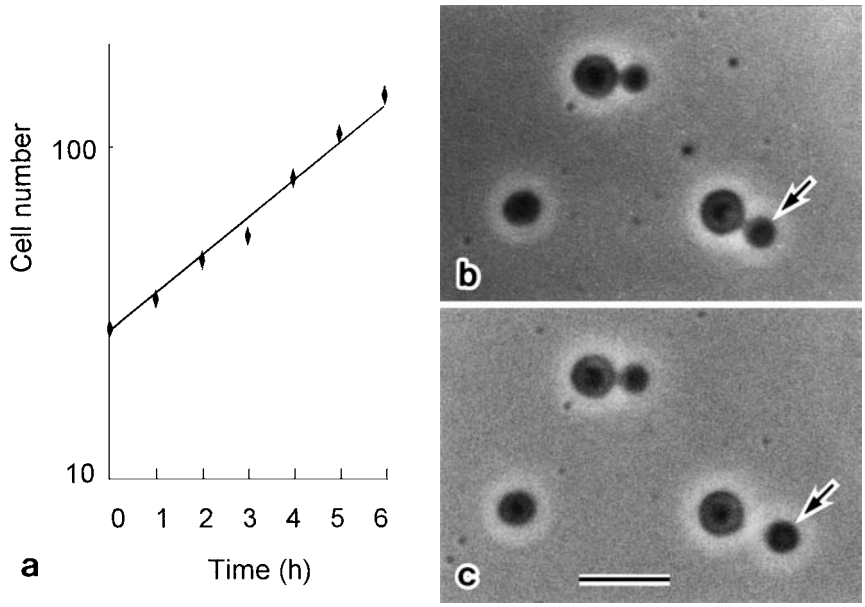


Fig. 5. Time-lapse experiments in petri-dish culture. (a) Cell proliferation during observation. Cells were found to be in an exponential growth phase. The cell indicated by arrow (b, time 0) separated after 3 min (c). Scale= 10 μm (b, c). Similar results were obtained in 11 other experiments using IFM 5808, 5844 and 41464 strains.

standard culture conditions (YPG medium, 24°C, and 150 rpm) was 132 ± 16 min (mean ± standard deviation, data from 3 independent experiments), the durations of the G1, S, G2, and M phases were estimated to be about 71, 18, 25, and 18 min, respectively (Fig. 4).

Time-lapse photomicrography in petri-dish culture

The doubling time of individual cells was measured by time-lapse photomicrography in petri-dish culture. The growth of the cells was exponential during the observation, and the culture was in a steady state (Fig. 5a). The data showing that the doubling time of the first division of the mother cells was not significantly different from those of second and third divisions (data not shown) support this assumption. The daughter cells were always born smaller than the mother cells (Figs. 5b-c). The doubling time of the daughter cells was 274 ± 27 min (15 measurements) and that of the mother cells was 132 ± 16 (32 measurements), indicating the doubling time of the daughter cells was about twice that of the mother cells. The doubling time of the whole population in petri-dish culture was 200 ± 46 min (data from 3 independent experiments).

Spindle pole body in interphase cells

Exponentially growing cells were examined by freeze-substitution electron microscopy. SPBs in interphase cells were on the outer nuclear envelope and dumbbell-shaped (Fig. 6). By serial sectioning of cells, SPBs were found to take these forms during the G1 and G2 phases. Each SPB was about 340 nm long with spheres on each end, each sphere being about 150 nm in diameter (additional data from a larger

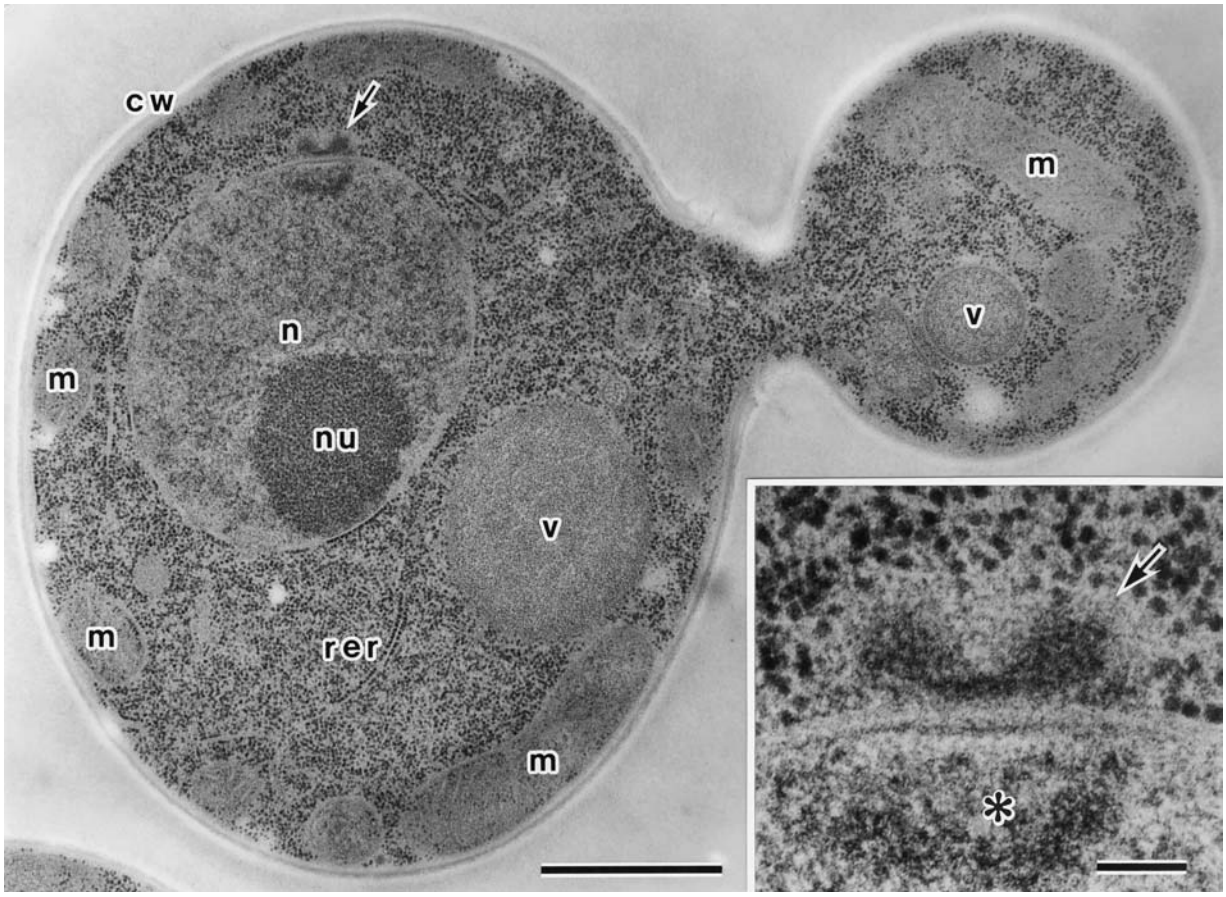


Fig. 6. Ultrathin section of a *C. neoformans* IFM 5844 cell at interphase. The SPB (arrow) was on the outer nuclear envelope and dumbbell-shaped. Similar images of the SPB were also observed in IFM 5808 and 45941 cells. cw, cell wall; m, mitochondrion; n, nucleus; nu, nucleolus; rer, rough endoplasmic reticulum; v, vacuole; and *, electron dense material. Scale= 1 μm, (inset, 100 nm).

number of observations will be published elsewhere).

Discussion

The doubling time of *C. neoformans* was found to be about 132 min, and the cell cycle was found to consist of 71-min G1, 18-min S, 25-min G2, and 18-min M phases under standard liquid culture conditions. It was also found, from the time-lapse experiment in petri-dish culture, that the doubling time of the daughter cells was nearly twice that of the mother cells. The reasons the population doubling time in petri-dish culture was longer than that in liquid cultures are not clear; however, it is presumed that the limited oxygen supply in a petri-dish is one reason, because the culture was left to stand during the experiment.

In *Saccharomyces cerevisiae*, the initiation of budding, the initiation of DNA synthesis, and SPB duplication occur simultaneously, all of which are known to be triggered by 'start' [10]. In *Exophiala dermatitidis*, pathogenic ascomycetous yeast, the initiation of budding and the initiation of DNA synthesis occur simultaneously, but SPB duplication occurs at the start of the G1 phase [11, 12]. In *C. neoformans*, the initiation of DNA synthesis starts before the initiation of budding (Figs. 2, 4). The SPB must have divided immediately after cell division, because a dumbbell-shape (Fig. 6) is observed throughout the interphase and this shape is considered to be a duplicated form [13]. That is, SPB duplication finishes before the initiation of budding and DNA synthesis. (The dynamics of SPB duplication from the standpoint of the cell cycle in *C. neoformans* will be published elsewhere). Thus, *C. neoformans* shows different cell cycle patterns from *S. cerevisiae* and *E. dermatitidis*. To understand the control mechanism for the growth of this pathogen, a cell cycle analysis including SPB dynamics would be helpful. Research in this direction is now in progress in our laboratory.

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References

1) Casedevall A, Perfect JR: *Cryptococcus neoformans*. ASM Press, Washington, DC, U.S.A., 1998.
2) Takeo K, Tanaka R, Miyaji M, Nishimura K: Unbudded G2 as well as G1 arrest in the stationary phase of the basidiomycetous yeast *Cryptococcus neoformans*. FEMS Microbiol Lett **129**: 231-236, 1995.
3) Ohkusu M, Hata K, Takeo K: Bud emergence is gradually delayed from S to G2 with progression of growth phase. FEMS Microbiol Lett **194**: 251-255, 2001.
4) Nishimura K, Miyaji M, Takeo K, Mikami Y, Kamei K, Yokoyama K, Tanaka R: IFM List of Pathogenic Fungi and Actinomycetes with Photomicrographs. 2nd edn. Culture Collection of Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University. Seibun-sha, Chiba, Japan, 1998.
5) Yamaguchi M, Biswas SK, Kita S, Aikawa E, Takeo K: Electron microscopy of pathogenic yeasts *Cryptococcus neoformans* and *Exophiala dermatitidis* by high-pressure freezing. J Electron Microsc **51**: 21-27, 2002.
6) Ohkusu M, Yamaguchi M, Hata K, Yoshida S, Nishimura K, de Hoog GS, Takeo K: Cellular and nuclear characteristics of *Exophiala dermatitidis*. Studies in Mycol **43**: 143-150, 1999.
7) Yamaguchi M, Biswas SK, Suzuki Y, Furukawa H, Takeo K: Three-dimensional reconstruction of a pathogenic yeast *Exophiala dermatitidis* cell by freeze-substitution and serial sectioning electron microscopy. FEMS Microbiol Lett **219**: 17-21, 2003.
8) Biswas SK, Yamaguchi M, Naoe N, Takashima T, Takeo K: Quantitative three-dimensional structural analysis of *Exophiala dermatitidis* yeast cells by freeze-substitution and serial ultrathin sectioning. J Electron Microsc **52**: 133-143, 2003.
9) Yamaguchi M, Shimizu M, Yamaguchi T, Ohkusu M, Kawamoto S: Repeated use of uranyl acetate solution for section staining in transmission electron microscopy. Plant Morphol **17**: 57-59, 2005.
10) Lew DJ, Weinert T, Pringle JR: Cell cycle control in *Saccharomyces cerevisiae*. In The Molecular and Cellular Biology of the Yeast *Saccharomyces*: Cell Cycle and Cell Biology (Pringle JR, Broach JR, Jones EW eds), pp. 607-695, Cold Spring Harbor Laboratory Press, Plainview, NY, 1997.
11) Yamaguchi M, Biswas SK, Suzuki Y, Furukawa H, Sameshima M, Takeo K: The spindle pole body duplicates in early G1 phase in a pathogenic yeast *Exophiala dermatitidis*: an ultrastructural study. Exp Cell Res **279**: 71-79, 2002.
12) Yamaguchi M, Kuwabara Y, Shimizu M, Furukawa H, Nishioka H, Takeo K: The spindle pole body of the pathogenic yeast *Exophiala dermatitidis*: variation in morphology and positional relationship to the nucleolus and the bud in interphase cells. Eur J Cell Biol **82**: 531-538, 2003.
13) Heath IB: Nucleus-associated organelles in fungi. Int Rev Cytol **69**: 191-221, 1981.