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Entamoeba invadens: Dynamics of DNA synthesis during differentiation from trophozoite to cyst

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ABSTRACT

The DNA dynamics which mediate conversion of uni-nucleate trophozoite into quadrinucleate cyst in *Entamoeba histolytica* is not well understood. Here, we have addressed this question in *Entamoeba invadens* (a model system for encystation) through a detailed time course study of the differentiation process. We combined flow cytometric analysis with the change in rate of thymidine incorporation and the number of nuclei per cell. Our data shows that during encystment the cell population passes through three phases: (1) Early phase (0–8 h); of rapid DNA synthesis which may correspond to completion of ongoing DNA replication. Bi-nucleated cells increase with concomitant drop in uni-nucleated cells. (2) Commitment phase (8–24 h); in which DNA synthesis rate slows down. Possibly new rounds of replication are initiated which proceed slowly, followed by mitosis at 20 h. After this the number of bi- and uni-nucleated cells gradually decline and the tri- and tetra-nucleated cells begin to increase. (3) Consolidation phase (24–72 h); in which the rate of DNA synthesis shows a small increase till 32 h and then begins to decline. The G2/M peak reappears at 48 h, showing that more rounds of DNA replication may be getting completed, followed by nuclear division. By 72 h the encystment is virtually complete. The bi-nucleated stage could be an intermediate both in the conversion of trophozoite to cyst and back. Our study provides a comprehensive view of DNA dynamics during encystation and excystation of *E. invadens*.

1. Introduction

Amoebiasis is the third commonest cause of death due to parasitic infection globally, and is a major health problem in developing countries (Stanley, 2003; Troll et al., 1997). It is acquired by ingestion of *Entamoeba histolytica* cysts, which get converted into the motile trophozoites in the ileo-cecal region of the intestine. Cysts are the infective stage, but so far it has not been possible to induce encystment in axenic *E. histolytica* trophozoites in culture. To study this differentiation process the model system used is *Entamoeba invadens*, the reptilian parasite which can encyst in culture (Avron et al., 1986; Sanchez et al., 1994; Vazquezdelara-Cisneros and Arroyo-Begovich, 1984). Encystation and excystation are among the simplest developmental processes in eukaryotes. Characterization of these processes may yield important information about the evolution of more elaborate developmental processes.

E. invadens trophozoites typically contain one nucleus whereas mature cysts contain four nuclei (Silberman et al., 1999). During the differentiation of trophozoite to cyst, an increase in the DNA content per cell is expected. Two rounds of DNA replication have been reported to occur during *E. invadens* encystment (Ganguly and Lohia, 2001; Kumagai et al., 1998), resulting in the tetra-nucleated cyst. However, no details of this process are known. Estimates of ploidy and the distribution of DNA in the typical cell-cycle stages in *Entamoeba* have been attempted previously, employing flow cytometry, and the results obtained have varied depending on the protocols used for fixing and staining the cells (Byers and Eichinger, 2005; Dvorak et al., 1995; Ganguly and Lohia, 2001; Mukherjee et al., 2008). The method employed by Dvorak's group eliminates the high natural fluorescence of *Entamoeba* trophozoites. With this method it was found that exponentially growing *E. invadens* trophozoites displayed G1, S and G2 phases in proportions typical of an eukaryotic cell population (Dvorak et al., 1995; Mitchison, 1971). However, cells prepared by other methods showed more atypical patterns, with apparent ploidies ranging from 1 to 16 N (Byers and Eichinger, 2005; Ganguly and Lohia, 2001). In addition to these inconsistencies, there is a paucity of information regarding the changes that take place in the uni-nucleated trophozoite during its conversion into a tetra-nucleate cyst. Here, we have attempted to address the dynamics of DNA synthesis through a detailed time course study at different stages of the life cycle from trophozoite to cyst and back to trophozoite. We prepared the cells for flow cytometry by Dvorak's method, and combined this analysis with the change in rate of thymidine incorporation and the number of nuclei per cell throughout the differentiation process.

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2. Materials and methods

2.1. Cell culture and maintenance

E. invadens strain IP-1 was obtained from the American Type Culture Collection and maintained at 25 °C in TYI-S-33 (Diamond, 1987) containing 15% heat inactivated adult bovine serum, 125 μL/100 ml streptomycin (Nicholas primal India limited) and 2% vitamin mix (Diamond, 1987).

2.2. Cyst induction and excystation

Log phase trophozoites grown in 50 ml flasks were chilled on ice for 10 min to remove the cells from the wall and harvested by centrifugation at 500*g* for 5 min at 4 °C. 5×10^5 trophozoites ml^{-1} were transferred into induction medium (LG): TYI medium was prepared without glucose and diluted to 2.12 times with water (Sanchez et al., 1994). Finally the medium was completed with 5% heat inactivated adult bovine serum, 2.6% vitamin mix (Diamond, 1987) and 125 μL/100 ml antibiotic. Cysts obtained in LG medium after 3 days were harvested and treated with 0.05% Sarkosyl (Sigma) to destroy the trophozoites (Sanchez et al., 1994). Typical spherical refractile cysts were observed by light microscopy and checked for staining with calcofluor. For excystation the cysts were further washed with phosphate-buffered saline (PBS), and inoculated in normal TYI-S-33 medium.

2.3. Flow cytometry

E. invadens cells were fixed and nuclei were isolated for flow cytometric analysis according to the protocol of Dvorak et al. (1995) with some modifications. Cells were chilled on ice for 10 min and harvested by centrifugation at 500*g* for 5 min at 4 °C. Pellet was resuspended in 700 μL solution "A" (1% Triton X-100, 0.75% NP-40, 125 mM $MgCl_2$ in PBS pH 7.2) and incubated on ice for 15 min, followed by addition of 300 μL solution "B" (125 mM $MgCl_2$ in PBS pH 7.2) and further incubated on ice for 15 min. Cells were centrifuged at 1500*g* for 5 min at 4 °C and nuclear pellet was washed three times with solution "B". Lysozyme (USB) was added (4 mg/ml) for the lysis of cyst wall. Finally, pellet was resuspended in 100 μL solution "B" and treated with 200 μg/ml RNase A (USB) at 37 °C for 60 min. RNase treatment was found to be crucial for obtaining high-resolution DNA content distributions. Propidium iodide (0.04 mg/ml, excitation 536 nm and emission at 617 nm) was added and incubation continued for another 20 min. Flow cytometry was performed with BD FACS Calibur (Becton–Dickinson) by using the filter FL-2. Data was analyzed using CellQuest Pro software.

2.4. Fluorescence microscopy

Cells were fixed in ethanol at −20 °C for 1 h. They were washed with PBS twice and thin smear was formed on cover slip, air dried and incubated with 5 μg/ml Hoechst 33342 (Invitrogen) in PBS for 30 min at 37 °C to stain the nuclei. The cover slip was mounted on glass slide with 25% glycerol containing *p*-phenylene diamine as anti bleach. Cells were observed using a Zeiss microscope with blue filter at magnification of 40×.

2.5. Thymidine incorporation

To measure the rate of DNA synthesis, cells were incubated with [H^3]-thymidine (10 μCi per 5×10^6 cells) for 1 h at 25 °C. Cells were chilled, harvested by centrifugation and precipitated with 10% chilled trichloroacetic acid (TCA) at 4 °C for 45 min. Precipitates were collected on GF/C glass microfibre filters (Whatman) and washed with 5% chilled TCA, followed by absolute ethanol. Filters were dried and incorporated radioactivity was determined after addition of scintillation fluid in a liquid scintillation counter (Packard).

3. Results

3.1. Flow cytometric analysis during encystation and excystation

Trophozoites of *E. invadens* in exponential growth displayed predominantly two major peaks representing G0/G1 and G2/M phases of the cell-cycle. The expected doubling of DNA content was seen in the G2/M peak compared with G0/G1 (Fig. 1A). The proportion of cells in G0/G1, S and G2/M phases was 66%, 7% and 12% respectively. When cells were transferred to LG medium for encystation, the same pattern was observed for the first 4 h (Fig. 1B). After that the G2/M peak gradually diminished and disappeared. This pattern was seen till 20 h (Fig. 1C–F). At 20 h the percentage of cells in G0/G1 and S phases was 86% and 5%, respectively, with no cells observed in G2/M. Further incubation in LG medium (24–72 h) lead to reappearance of the G2/M peak (8%) at 48 h, followed by its decline at 72 h (Fig. 1G–I). The kinetics was not followed beyond 72 h by which time the process of encystment was virtually completed. When Sarkosyl treated mature cysts were inoculated in the normal growth medium for excystment into trophozoites, a single broad peak corresponding to G0/G1 phase was seen at 0 h (Fig. 2A). Within 24 h, (Fig. 2B) 8% of the cell population was observed in G2/M phase, while the typical pattern of exponentially growing trophozoites was observed by 48 h (Fig. 2C).

3.2. Number of nuclei per cell during encystation and excystation

Samples were removed at regular intervals after transferring to encystation medium, and nuclei were stained with Hoechst dye. The number of nuclei per cell was counted using a fluorescence microscope, and the percentage of cells with one, two, three, four or more nuclei were recorded during the differentiation process. The same was repeated when cysts were placed in growth medium for excystment. Up to 300 cells were counted at each time point and care was taken not to introduce errors in the determination of nuclear number due to superposition of nuclei.

During encystation, (Fig. 3A) just before shifting to LG medium, the culture contained, on an average (three independent measurements), 95% uni-nucleated and 5% bi-nucleated cells, but no tri- and tetra-nucleated cells were observed. After shifting to LG medium the number of bi-nucleated cells began to increase. By 12–16 h the bi-nucleated cells increased to 10–20% of total cells, and the uni-nucleated cells correspondingly decreased to 70–80%. Beyond that (24–72 h), there was gradual decrease in uni- and bi-nucleated cells, with significant increase in tri- and tetra- nucleated cells. Sometimes a few 5- and 6-nucleated cells were also seen. After 72 h, 40% cells remained uni-nucleated, out of which 10% cells were cysts as shown by Sarkosyl resistance and staining with calcofluor. On an average, of the original cell population 30% remained as trophozoites and 70% cells were converted to cysts.

During excystation, (Fig. 3B) at 0 h in normal TYI-S-33 medium, the distribution of cysts with respect to number of nuclei per cyst was as follows: cysts with uni-nucleate (10%), bi-nucleate (18%), tri-nucleate (20%) and tetra-nucleate (52%). This pattern continued till 8–12 h, beyond which the number of tri- and tetra-nucleated cells began to drop. The bi-nucleated cells showed a slight increase at 12–16 h. The first significant increase in the number of uni-nucleated cells (32%) occurred at 24 h with concomitant drop in tri- and tetra-nucleated cells (13% and 37%, respectively). By