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Short communication

Evaluation of drugs and stationary growth on the cell cycle of *Giardia intestinalis*

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ABSTRACT

We examined the effect of aphidicolin, colchicine, demecolcine, fluorouracil, hydroxyurea, and nocodazole, as well as nutrient deprivation on the *Giardia intestinalis* cell cycle. Aphidicolin was the only drug that was able to block the cell cycle at a specific stage (G1/S), and permit cells to resume growth at a high rate upon its removal. Nutrient deprivation resulted in a portion of G2/M cells completing mitosis and cytokinesis in synchrony during the recovery period, but this synchrony was shortly lost and a sample containing a predominance of G1 cells could not be obtained. Flow cytometry analysis of normal and untreated *Giardia* cultures showed the occasional appearance of a small percentage of cells with a DNA content of 16C, which is twice the DNA content of G2 cells. However, this 16C peak is larger and more frequently observed in drug-treated *Giardia*. These 16C are likely produced from endoreplication of 8C/G2 cells, and we propose that they represent a pre-encystation stage that is induced by drug treatments and other stressors.

Giardia intestinalis is among the most commonly reported intestinal protozoa in the world [1] with infections documented in humans and over 40 species of animals [2]. The lifecycle of *Giardia* alternates between the motile trophozoite form and the infectious dormant cyst form. The proliferation of *Giardia* trophozoites during an active infection, and the restriction point for the differentiation of trophozoite to cyst are processes dependent on the tight regulation of the cell cycle [3]. To carry out molecular and genetic studies of the cell cycle, it is necessary to obtain sufficient numbers of cells representative of each cell cycle stage. Two current methods are currently available to obtain cell cycle synchrony in *Giardia* trophozoite cultures. In one procedure, *Giardia* cells are treated with 5 μg/mL (14.8 μM) of aphidicolin for 6 h [4]. In the other procedure, cells are treated with 100 nM nocodazole for 2 h followed by incubation with 6 μM aphidicolin for 6 h [5]. However, a recent study showed that aphidicolin can cause double-stranded DNA breaks in *Giardia* trophozoites and hence, may complicate cell cycle studies when this drug is used to synchronize *Giardia* cultures [6]. In this report we further studied the effects of aphidicolin and nocodazole, and explored the effect of four other drugs and stationary phase growth on the *Giardia* cell cycle. Aphidicolin, fluorouracil and hydroxyurea are drugs that block the cell cycle at G1/S or S-phase in other organisms by inhibiting DNA replication. By comparison, colchicine, demecolcine, and nocodazole are expected to block the cell cycle at G2/M or M-phase by inhibiting the mitotic spindles.

We first examined the distribution of cells within the cell cycle in *Giardia* trophozoite cultures without drug treatments. Flow cytometry (FC) based on the cellular DNA content of *Giardia* cultures at the exponential and stationary phases of growth showed two major peaks as well as a much smaller third peak in some of these cultures (Fig. S1). The assignment of these peaks to particular cell cycle stages has been a matter of dispute. In previous flow cytometry studies on *Giardia* trophozoites, Hoyne et al. [7] and Gosh et al. [8] designated peak ii as G1 cells and peak iii as G2/M cells. Bernander et al. [3] used *E. coli* cells containing multiple copies of chromosomes to generate a plot of DNA content *versus* the position of each peak observed on a FC histogram. This plot was used to extrapolate the DNA content of the three peaks found in the FC histogram for *Giardia* trophozoites assayed in parallel samples. By comparison, we used a more direct approach to determine the identity of the three *Giardia* peaks by mixing *Giardia* trophozoites with haploid and diploid strains of *Saccharomyces cerevisiae* together in a single sample for FC analysis (Fig. 1A). The haploid yeast strain BY4741 has 12 Mb of DNA in G1 cells and 24 Mb in G2/M cells, whereas the diploid strain MCY297 has 24 Mb of DNA in G1 and 48 Mb of DNA in G2/M cells. When a sample containing *Giardia* cells mixed with both strains of yeast was analyzed, the second peak of *Giardia* cells co-localized with the G2/M peak of the diploid yeast (Fig. 1A, yeast + *Giardia* panel). As the *Giardia* genome is ~12 Mb

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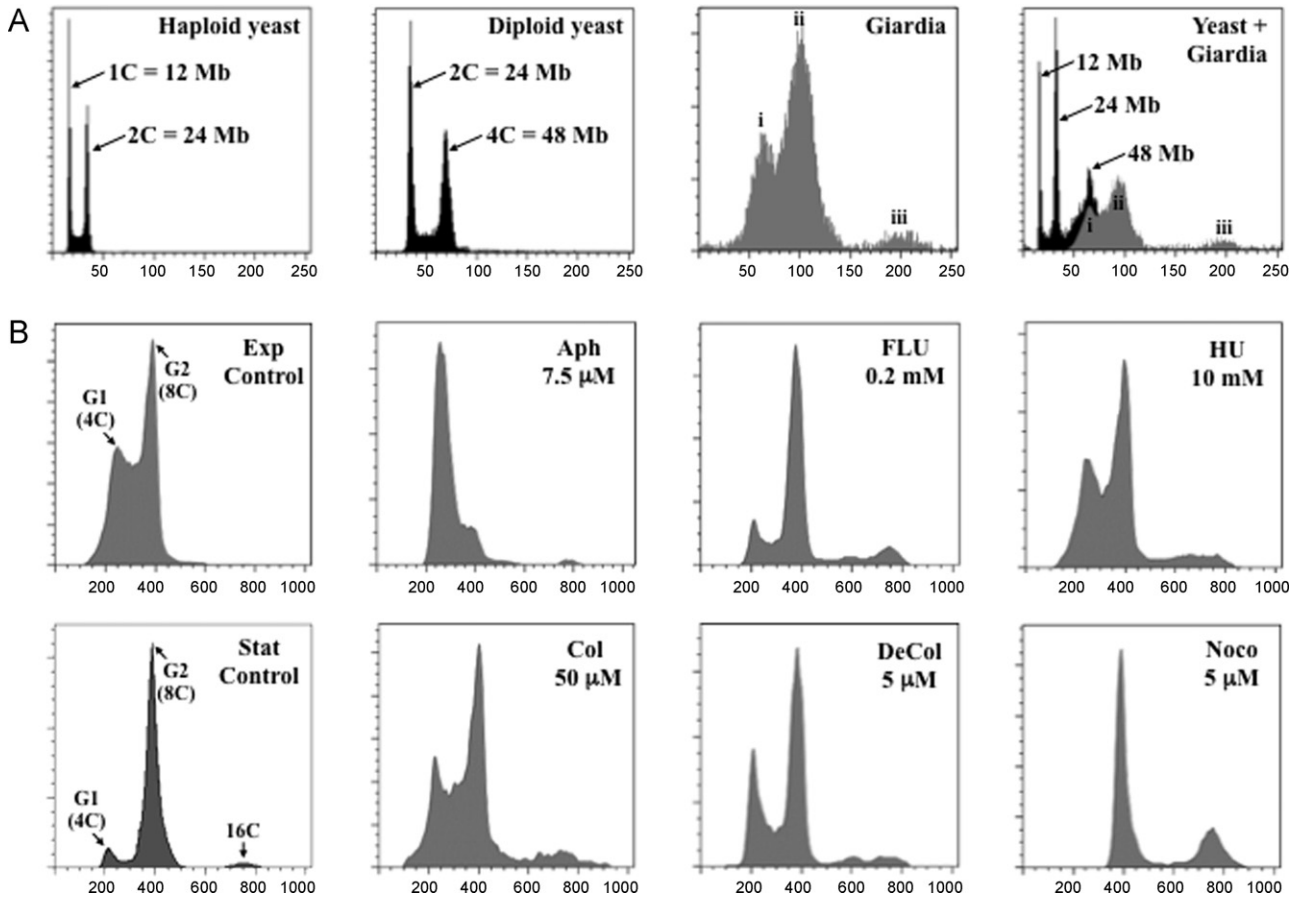

Fig. 1. Flow cytometry analysis of *Giardia* trophozoite cultures. (A) Two strains of *S. cerevisiae*, BY4741 (Haploid) and MCY297 (Diploid), were grown at 37 °C in YPD media to an OD_{660} of 0.5–0.6. The cells were collected by centrifugation and resuspended in 1 mL of 70% ethanol followed by incubation at room temperature for 15 min [19]. Immediately prior to flow cytometry, the fixed yeast cells were collected by centrifugation, washed 1× with H_2O, and resuspended in RNase solution A (0.2 mg/mL RNase A in 50 mM Tris–HCl, pH 8). After a 2–4 h incubation at 37 °C, the cells were pelleted, and resuspended in a proteinase K solution (2 mg/mL proteinase K in 50 mM Tris–HCl, pH 7.5). After a 30–60 min incubation at 50 °C, the cells were pelleted and resuspended in yeast flow cytometry buffer (200 mM NaCl, 78 mM $MgCl_2$, 200 mM Tris–HCl, pH 7.5). A 100 μL aliquot of cells was added to 1 mL of 1 μM SYTOX Green in 50 mM Tris–HCl (pH 7.5) and then sonicated for 5 s at low intensity (level 2–3) in a Sonic Dismembrator model 100 (Fisher Scientific). For the preparation of the sample mixed with the *Giardia* cells in the peak calibration experiment, yeast cells were resuspended in PBS after proteinase K treatment, sonicated, and stained by the addition of SYTOX Green (in DMSO) to 5 μM on the day of the flow cytometry experiment. The *y*-axes of the flow cytometry profiles are normalized to the highest peak. (B) *Giardia* cells were prepared for flow cytometry as described by Bernarder et al., [3] except that SYTOX Green (Invitrogen) was used as the nucleic acid stain. Immediately prior to flow cytometry, 4 μL of 50 μM SYTOX Green was added to each 200 μL sample containing 1×10^6 cells (1 μM final concentration). The stained cells were analyzed on a either an FACS Aria 3 (BD Biosciences), FACS Canto (BD Biosciences) or Cytomics FC 500 (Beckman–Coulter) flow cytometer. Cell cycle analysis of the flow cytometry data was performed with the FlowJo Analysis Software 7.2.2 (Tree Star, Inc.). The histogram represents SYTOX green fluorescence (DNA content) on the *x*-axis and cell count (number of events) on the *y*-axis. The y-axes of the flow cytometry profiles are normalized to the highest peak.

and trophozoites are tetraploid (4N) with 2C of DNA (the haploid amount of DNA) in each of two identical nuclei [3], the 48 Mb peak (peak i) in the *Giardia* flow cytometry histogram represents G1 cells, while peak ii of *Giardia* cells are post-replicated 8N cells at G2/M.

The third peak of *Giardia* cells in the flow cytometry histogram have approximately twice as much DNA as the G2/8C *Giardia* cells in peak ii (Fig. 1A). As we excluded doublet cells by applying the necessary gating parameters to the flow cytometry data (Fig. S2), we propose that peak iii contain singlet 16C trophozoites. Treatment of *Giardia* cultures with drugs also induces the appearance of the third peak to varying extents (Fig. 1B). The association of peak iii with drug treatments suggests that the 16C cells may be a response to stress. Previous studies by Reiner et al. [4] showed that G2 is the restriction point for initiating the encystation process to produce 16C cysts. The increased expression of cytosolic HSP70 at the mRNA level [9] and at the protein level [10] in *Giardia* during encystation suggest that this process may be partially linked to a cellular stress response. However, the *Giardia* cultures used in our study were grown in normal TYI-S-33 medium [11], which do not have necessary conditions, such as the pH or the type of bile, for trophozoites to complete the encystation process [12]. Furthermore, our microscopic examination of *Giardia* cultures containing the third peak in flow cytometry histogram detected only two nuclei per trophozoites and no cysts (data not shown). Our observations suggest that there is a small population (1–5%) of 16C cells produced in normal *Giardia* cultures from endoreplication of G2/8C trophozoites. In plants and animals, one role of endoreplication is to help cells adapt and protect themselves from physiological and environmental stressors [13]. The most widely used method to induce *Giardia* encystation *in vitro* is a 2-step process that involves incubation of trophozoites for 2 days in a pre-encystation medium followed by incubation in an encystation medium for another 2 days [12]. Thus, the completion of encystation is a long process and likely requires the temporal and spatially regulated activation of multiple cellular events. When *Giardia* cultures are exposed to drugs that disrupt the cell cycle, we speculate that an induction of the endoreplication of G2 cells may be an attempt by *Giardia* to enter the encystation pathway. Since encystation cannot be completed in the TYI-S-33 medium and the drug treatments may have caused irreversible cellular damage, these endoreplicated cells remain stalled at an early stage within the encystation pathway in these cultures.