

# Cell Cycle Synchrony in *Giardia intestinalis* Cultures Achieved by Using Nocodazole and Aphidicolin<sup>∇</sup>

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***Giardia intestinalis* is a ubiquitous intestinal protozoan parasite and has been proposed to represent the earliest diverging lineage of extant eukaryotes. Despite the importance of *Giardia* as a model organism, research on *Giardia* has been hampered by an inability to achieve cell cycle synchrony for in vitro cultures. This report details successful methods for attaining cell cycle synchrony in *Giardia* cultures. The research presented here demonstrates reversible cell cycle arrest in G<sub>1</sub>/S and G<sub>2</sub>/M with aphidicolin and nocodazole, respectively. Following synchronization, cells were able to recover completely from drug treatment and remained viable and maintained synchronous growth for 6 h. These techniques were used to synchronize *Giardia* cultures to increase the percentages of mitotic spindles in the cultures. This method of synchronization will enhance our ability to study cell cycle-dependent processes in *G. intestinalis*.**

*Giardia intestinalis* is a ubiquitous intestinal protozoan parasite causing disease in humans and animals worldwide (1, 11). In developing countries, diarrheal disease is responsible for 80% of the deaths of children under 2 years of age (21), and *Giardia* is one of the major causes of this condition. As a diplomonad, *Giardia* has been proposed to represent the earliest diverging lineage of extant eukaryotes, based on single rRNA and single and/or concatenated protein phylogenies developed by considering an archaeal out-group (2, 3, 5, 15, 23), making it a valuable organism for studying the evolution of biological processes in all eukaryotes. Characteristic of the order Diplomonadida, *Giardia* trophozoites contain two nuclei that remain separate during mitosis, with each daughter cell inheriting one copy of each parental nucleus (19). The trophozoite form, which attaches to the small intestine of the host, has a tetraploid (4N) DNA content in G<sub>1</sub> since each nucleus is 2N (4). Following a round of DNA synthesis, each G<sub>2</sub> nucleus is 4N, making the cell 8N. According to previous flow cytometry results, actively growing *Giardia* cultures spend the majority of the cell cycle in the G<sub>2</sub>/M phase and significantly less time in the G<sub>1</sub> and S phases (4); in contrast, many tissue culture cells display a lengthy G<sub>1</sub> phase. Until recently, an inability to synchronize in vitro *Giardia* cultures to any degree has severely hampered the ability of researchers to study cell cycle-dependent processes (16, 20).

This work demonstrates successful cell cycle arrest by using nocodazole, a microtubule-destabilizing drug that leads to the depolymerization of spindle microtubules in *Giardia* (6, 20). A brief nocodazole treatment resulted in cells arrested early in mitosis or at the end of G<sub>2</sub>, presumably by the activation of a mitotic spindle checkpoint (22). G<sub>2</sub> arrest using nocodazole was combined with G<sub>1</sub> arrest using aphidicolin, a drug that

presumably acts through the inhibition of polymerase-dependent DNA synthesis (8, 12, 14, 25). By combining these two treatments, we were able to effectively synchronize *Giardia* cultures while maintaining cell viability. These synchronization methods were used to enrich cultures with mitotic spindles at the M phase. Moreover, these methods will be a valuable tool for studying other aspects of *Giardia* biology such as encystation, the time in the life cycle when the trophozoite transforms into an infectious cyst.

## MATERIALS AND METHODS

**Culture conditions and growth curves.** *G. intestinalis* trophozoites, strain WBC6, ATCC 50803, were grown in modified TYI-S-33 medium with adult bovine bile (catalog no. B9433; Sigma) (10). Cultures were maintained in 15-ml plastic screw-top tubes (Fisher Scientific) at 37°C. Growth curves were constructed by counting cells with a hemacytometer at the time points specified in Fig. 4. Prior to counting, the cells were placed on ice for 15 min to detach the cells.

**Synchronization.** For synchronization studies, confluent cultures containing approximately  $2.5 \times 10^6$  cells/ml were iced for 20 min to detach cells. The experiments were conducted in 8-ml polystyrene tubes (Falcon tube no. 35-2027). The appropriate number of 8-ml cultures were started by adding  $\sim 2.5 \times 10^6$  cells to a final volume of 6 ml of modified TYI-S-33 medium (10) for a final concentration of  $\sim 4.15 \times 10^6$  cells/ml, and the cultures were incubated for anywhere from 24 to 36 h until they were approximately 80% or less confluent and were in log phase of growth. The old medium containing detached and dead cells was decanted and replaced with fresh 37°C growth medium and 100 nM nocodazole (catalog no. M1404; Sigma), and the cultures were incubated at 37°C. After 2 h the medium containing the nocodazole, as well as any unattached cells, was again decanted and replaced with fresh 37°C growth medium and 6  $\mu$ M aphidicolin (catalog no. 10797; Fluka). The nocodazole and aphidicolin were both dissolved in dimethyl sulfoxide (DMSO) and kept at  $-20^\circ\text{C}$ . Control cultures received an amount of DMSO equivalent to the amount of the nocodazole- and aphidicolin-DMSO solutions. After 6 h the medium was decanted and replaced with fresh 37°C growth medium for the prescribed recovery times.

**Cell fixation and preparation for flow cytometry analysis.** Following the individual treatments (i.e., nocodazole treatment, aphidicolin treatment, and recovery), the cultures were placed on ice for 15 to 20 min to detach cells. The cells were pelleted by centrifugation at  $800 \times g$  for 5 min, the supernatant was aspirated, and the pellet was washed twice in 2 ml of HEPES-buffered saline (150 mM NaCl, 5 mM KCl, 1 mM MgSO<sub>4</sub>, 10 mM HEPES, pH 7.4). For fixation, the cells were resuspended in 300  $\mu$ l of HEPES-buffered saline and 700  $\mu$ l of ice-cold 100% EtOH was added drop by drop while the cell suspension was subjected to a gentle vortex. At this point, the cells could be stored at 4°C indefinitely. The

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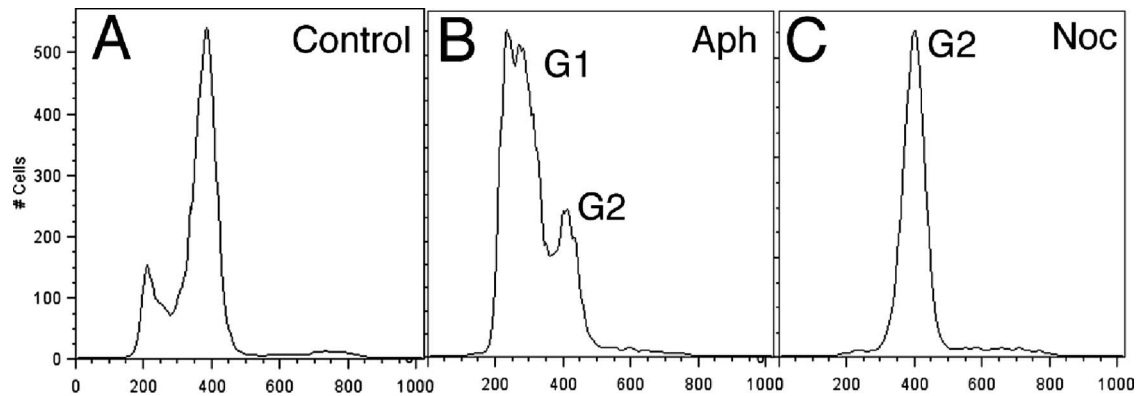


FIG. 1. Arrest of *Giardia* cell cycle with aphidicolin and nocodazole. (A and B) Subjecting a control culture with a 5:1 ratio of  $G_2$  to  $G_1$  cells (A) to treatment with  $6 \mu\text{M}$  aphidicolin (Aph) for 6 h results in very loose cell cycle arrest in  $G_1$ , with a significant number of cells in  $G_2$  (B). (C) After the removal of aphidicolin, cultures were exposed to  $100 \text{ nM}$  nocodazole (Noc) for 2 h, resulting in very tight  $G_2$  arrest.

cultures were then centrifuged, the pellet was rinsed in  $50 \text{ mM}$  Na citrate (Sigma) and resuspended in a  $0.5\text{-ml}$  volume of  $50 \text{ mM}$  Na citrate containing fresh RNase A at  $0.1 \text{ mg/ml}$ , and the suspension was incubated overnight at  $4^\circ\text{C}$ . Directly prior to flow cytometry analysis, a  $0.5\text{-ml}$  volume of  $50 \text{ mM}$  Na citrate containing  $10 \mu\text{M}$  Sytox green (Sigma) was added for a final Sytox green concentration of  $5 \mu\text{M}$ . Flow cytometry was performed on a Beckman Coulter EPICS XL flow cytometer, and the data were analyzed with FlowJo software (Tree Star Inc., Ashland, OR).

**Viability assay.** The viability of the control and treated *Giardia* cultures was determined using the adsorption indicator phloxine B (catalog no. 28550; Fluka). Samples of cultures were stained to a final concentration of  $2.5 \text{ g}$  of phloxine B/liter, and cells were counted on a light microscope. Dead cells stain bright pink, while live cells remain clear.

**Immunolocalization.** Trophozoites were fixed in the culture tubes with  $1\%$  paraformaldehyde for  $10 \text{ min}$ , centrifuged, washed with PEM buffer ( $100 \text{ mM}$  PIPES [piperazine- $N,N'$ -bis(2-ethanesulfonic acid)],  $1 \text{ mM}$  EGTA,  $0.1 \text{ mM}$   $\text{MgSO}_4$ ), and attached to poly-L-lysine-coated coverslips. Cells were permeabilized in  $0.1\%$  Triton X-100 for  $15 \text{ min}$ . Coverslips were washed with PEM buffer and blocked for  $30 \text{ min}$  in PEMBALG (PEM with  $1\%$  bovine serum albumin,  $0.1\%$  sodium azide,  $100 \text{ mM}$  lysine, and  $0.5\%$  cold-water fish skin gelatin [Sigma, St Louis, MO]). Microtubules were visualized by incubating coverslips with the monoclonal  $\alpha$ -tubulin antibody TAT1 (26) diluted  $1:75$  in PEMBALG at room temperature overnight. The coverslips were then rinsed and incubated with Alexa Fluor 555 (Molecular Probes, Eugene, OR) diluted  $1:50$  in PEMBALG at room temperature for  $2 \text{ h}$ . The coverslips were then rinsed in PEMBALG and PEM before being mounted with ProLong AntiFade with DAPI (4',6'-diamidino-2-phenylindole; Molecular Probes).

**Fluorescence deconvolution microscopy.** Images were collected using SoftWorX image acquisition software (Applied Precision, Issaquah, WA) and an Olympus IX70 wide-field inverted fluorescence microscope with an Olympus Uplan apochromat  $100\times$  oil-immersion objective (numerical aperture,  $1.35$ ) and a Photometrics charge-coupled device CH350 camera cooled to  $-35^\circ\text{C}$  (Roper Scientific, Tucson, AZ). Serial sections were acquired at  $0.2\text{-}\mu\text{m}$  intervals, and data stacks were deconvolved using the SoftWorX deconvolution software. For printing purposes, two-dimensional projections were created from the three-dimensional data sets by using the DeltaVision image analysis software (Applied Precision).

## RESULTS

Figure 1A shows flow cytometry-determined DNA distributions for an untreated control population of *Giardia* cells, with two prominent peaks. Based on previous flow cytometry results (4), we interpret the first peak to represent cells in  $G_1/S$  ( $4N$ ) and the second to represent cells in  $G_2/M$  ( $8N$ ). Aphidicolin is a common synchronizing agent for eukaryotic cells that acts by blocking the cell cycle in the  $G_1/S$  phase (18). Initial attempts at synchronizing *Giardia* cultures in our laboratory indicated

that exposure to the high concentrations of aphidicolin required to effectively attain arrest at  $G_1/S$  caused cell death shortly after the removal of the drug (data not shown). We determined that the highest concentration of aphidicolin that *Giardia* cultures could tolerate was  $6 \mu\text{M}$ , and  $6 \text{ h}$  was the optimal incubation period, allowing the cells to remain viable. Figure 1B shows the profile of a culture following the  $6\text{-h}$ ,  $6 \mu\text{M}$  aphidicolin treatment, with the accumulation of cells in  $G_1/S$ ; however, a significant number of cells remained in  $G_2$  (Fig. 1B). In initial experiments using nocodazole, a microtubule-destabilizing drug, cells were arrested in  $G_2$ ; however, the removal of the drug did not lead to a noticeable synchronization of the cell cycle. In an attempt to produce an effective synchronization protocol, we developed a two-step procedure combining both drugs. The addition of  $100 \text{ nM}$  nocodazole following a  $6\text{-h}$ ,  $6 \mu\text{M}$  aphidicolin treatment resulted in efficient cell arrest, with close to  $100\%$  of the cells arresting in  $G_2$  (Fig. 1C). In order to arrest cells at  $G_1/S$  and avoid the possibility of an irreversible disruption of microtubule arrays by nocodazole, we reversed the order of the treatments, resulting in arrest in  $G_2$  followed by  $G_1/S$ . The addition of  $100 \text{ nM}$  nocodazole to a log-phase culture for  $2 \text{ h}$  resulted in cell cycle arrest, eliminating the first peak from the control (Fig. 2A) and causing all cells to accumulate in the stage represented by the second peak, identified as  $G_2$  (Fig. 2B). This drug concentration leads to the depolymerization of spindle microtubules (20), arresting cells early in mitosis or at the end of  $G_2$  presumably by the activation of a mitotic spindle checkpoint (22). Upon the removal of nocodazole-containing medium and the addition of fresh medium containing  $6 \mu\text{M}$  aphidicolin for  $6 \text{ h}$ , the cells arrested at the position of the first peak (Fig. 2C). The nocodazole treatment could then be repeated, and the cells arrested in  $G_2/M$  again (Fig. 2D). Using drugs to reversibly arrest the cell cycle and toggle between  $G_2$  and  $G_1$  and back to  $G_2$  while monitoring changes in DNA contents in the cell population confirmed the identities of the respective  $G_1$  and  $G_2$  peaks as described by Bernander et al. (4).

In order to ascertain how quickly the cell cycle was reestablished after our dual drug treatment and determine subsequent cell viability, we performed a recovery time course experiment following treatment with nocodazole and aphidicolin. Expo-

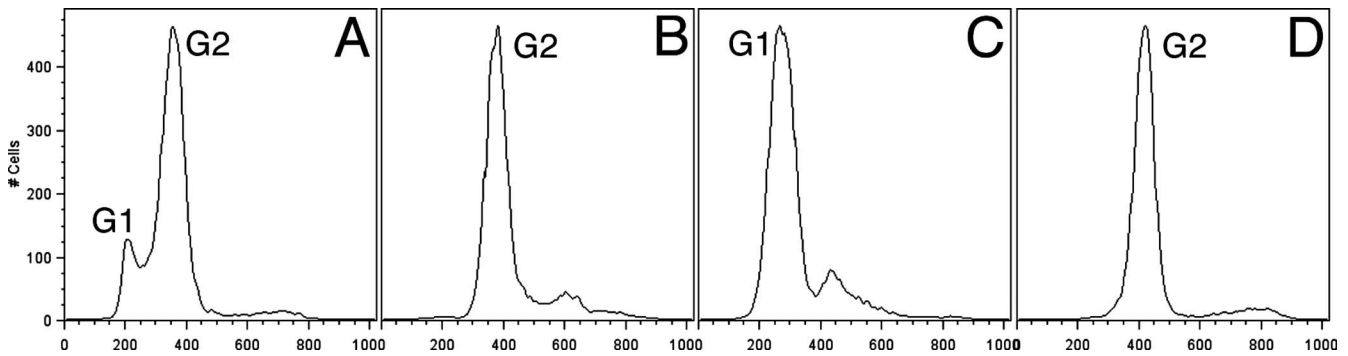


FIG. 2. Arrest of *Giardia* cell cycle with nocodazole and aphidicolin. (A) Starting culture with a 5:1 ratio of G<sub>2</sub> to G<sub>1</sub> cells. (B) Arrest in G<sub>2</sub> after exposure to medium containing 100 nM nocodazole for 2 h. (C) The removal of nocodazole and exposure to medium containing 6 μM aphidicolin for 6 h result in G<sub>1</sub> arrest. (D) Cultures are returned to G<sub>2</sub> by another treatment with 100 nM nocodazole. Data were collected as described above and gated according to a normal flow cytometry procedure using FlowJo software (Tree Star Inc., Ashland, OR).

sure to 100 nM nocodazole for 2 h followed by treatment with 6 μM aphidicolin for 6 h resulted in cell cycle arrest first in G<sub>2</sub>/M and then in G<sub>1</sub> (Fig. 3B and C). Following the removal of nocodazole-containing medium and the addition of medium containing 6 μM aphidicolin, cells arrested in G<sub>1</sub> (Fig. 3C). After the removal of aphidicolin medium and replacement with fresh medium, the cells were allowed to recover under normal growth conditions. The cells remained synchronously in G<sub>2</sub> for 3.0, 3.5, and 4.0 h (Fig. 3D to F). By 4.5 h, the cells began to go through mitosis and to accumulate in G<sub>1</sub> (Fig. 3G). After 5.0, 5.5, and 6.0 h of recovery, the cells continued to accumulate in G<sub>1</sub> (Fig. 3H to J). After 12 h of recovery, the cells had returned to the normal 5:1 ratio of G<sub>2</sub> to G<sub>1</sub> cells, as seen in the control sample (Fig. 3L). Data were collected as described in Materials and Methods and gated according to the normal flow cytometry procedure using FlowJo software (Tree Star Inc., Ashland, OR).

In addition to the cytometry studies, cell viability was checked and growth curves were constructed for cultures prior

to and following drug treatments. Cell viability before and after the treatments was assessed by staining the dead cells in cultures with phloxine B and tallying these cells relative to the number of live cells (Table 1). The number of dead cells in the starting culture decreased after pretreatment due to the dead cells' being decanted along with the detached cells prior to the addition of fresh nocodazole-containing medium. Following the 2-h nocodazole treatment, there was only a small increase in the number of dead cells, while following both the nocodazole and aphidicolin treatments, 90% of the cells remained viable to enter the recovery time course. Figure 4A shows two growth curves. The control curve represents the cultures from the time they were started until the drug treatments began at 36 h and shows normal log-phase growth. The recovery curve begins with time point zero as the removal of the final aphidicolin treatment and the addition of fresh growth medium. The release from cell cycle arrest was followed by a visible increase in cell division at approximately 6 h, which corresponded to the increase in G<sub>1</sub> cells seen in Fig. 3. As recovery continued,

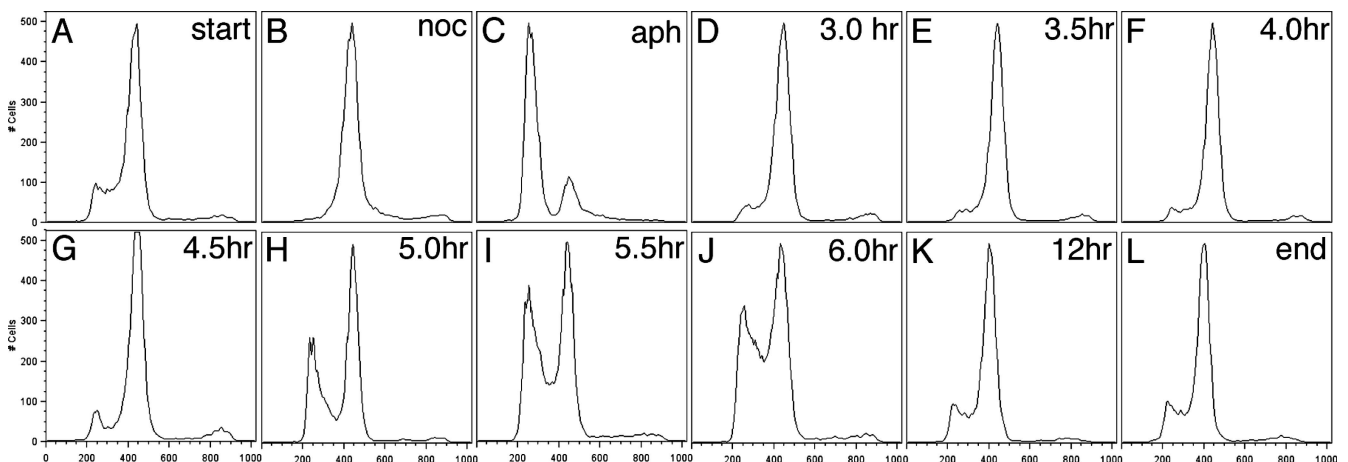


FIG. 3. Recovery following cell cycle arrest in *Giardia* cultures. (A and B) *Giardia* cultures propagated under normal growth conditions (A) and exposed to 100 nM nocodazole (noc) for 2 h arrest in G<sub>2</sub> (B). (C) Following the removal of nocodazole-containing medium and the addition of medium containing 6 μM aphidicolin (aph), cells arrest in G<sub>1</sub>. (D to F) After the removal of aphidicolin medium and replacement with fresh medium, the cells are allowed to recover under normal growth conditions for 3.0, 3.5, and 4.0 h. (G) By 4.5 h, the cells begin to go through mitosis and accumulate in G<sub>1</sub>. (H to J) After 5.0, 5.5, and 6.0 h of recovery, the cells continue to accumulate in G<sub>1</sub>. (L) After 12 h of recovery, the cells have returned to the normal 5:1 ratio of G<sub>2</sub> to G<sub>1</sub> cells as seen in the control sample.

TABLE 1. Results of the viability assay<sup>a</sup>

Culture status	% of viable cells
Starting culture .....	94.06 ± 1.89
Pretreated culture .....	97.56 ± 1.51
Culture treated with nocodazole for 2 h .....	95.76 ± 3.76
Culture treated with nocodazole for 2 h and aphidicolin for 6 h .....	90.00 ± 3.11

<sup>a</sup> Cell viability was documented using the adsorption dye phloxine B before the treatment began (starting culture), after the decanting of the growth medium prior to treatment (pretreated culture), following the nocodazole treatment, and following the nocodazole and aphidicolin treatments. Values are means ± standard deviations.

the cells continued to divide, albeit more slowly, until lag phase was reached by 36 h after treatment. Figure 4B shows the two curves plotted as one continuous line to represent the data in terms of time, from 0 to 84 h.

Light microscope evaluation at the 4.5-, 5.0-, and 5.5-h recovery time points demonstrated a visible increase in mitotic spindles compared to those in control cultures. Figure 5A and B shows mitotic spindles in *Giardia* cells undergoing metaphase and anaphase, respectively. Figure 6 shows that the number of spindle-containing cells relative to the number in control samples increased more than 100-fold between 4.5 and 5.0 h of recovery. These increases corresponded to the initial appearance of the G<sub>1</sub> peak (Fig. 3G) and the increase in G<sub>1</sub> peak height after 5.0 h of recovery (Fig. 3H and 4). The flow cytometry-determined DNA distributions indicate that there was still an increase in the number of cells entering G<sub>1</sub> compared to the number of such cells in the control after 5.5 h of recovery. However, the number of spindles increased only 60-

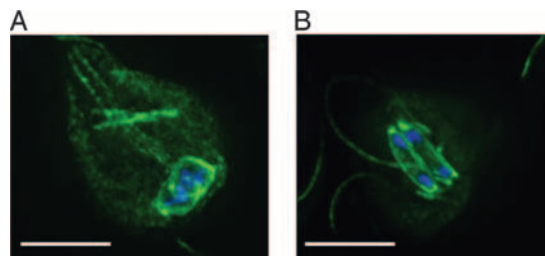


FIG. 5. Mitotic spindles in *Giardia*. Spindles were labeled with the TAT1 antitubulin antibody (26) and an Alexa 488-conjugated goat anti-mouse secondary antibody (Molecular Probes) in metaphase (A) and anaphase (B) after 4.5 (A) and 5.0 (B) hours of recovery from nocodazole and aphidicolin treatments. Nuclei were stained with DAPI (blue). Bars, 5 μm.

fold over that in the control. This result suggests that the wave of mitosis was complete and the cells were accumulating in G<sub>1</sub>.

Through the course of this work, we observed that previously published flow cytometry DNA distributions of *Giardia* trophozoites showed wide unresolved peaks which were difficult to interpret (7, 17, 21). Recent advances in *Giardia* flow cytometry protocols (4) greatly improved peak resolution but were not readily reproducible in our laboratory. Therefore, we developed a simple and reproducible flow cytometry protocol utilizing ethanol as a fixative and Sytox green as a fluorescent DNA dye. This protocol simplifies flow cytometry for *Giardia* cultures and enhances the resulting data by providing clean DNA distributions with well-differentiated peaks. As observed in Fig. 1 and 2, the locations of the G<sub>1</sub> and G<sub>2</sub> peaks shifted slightly along the x axis among the various experiments. This is

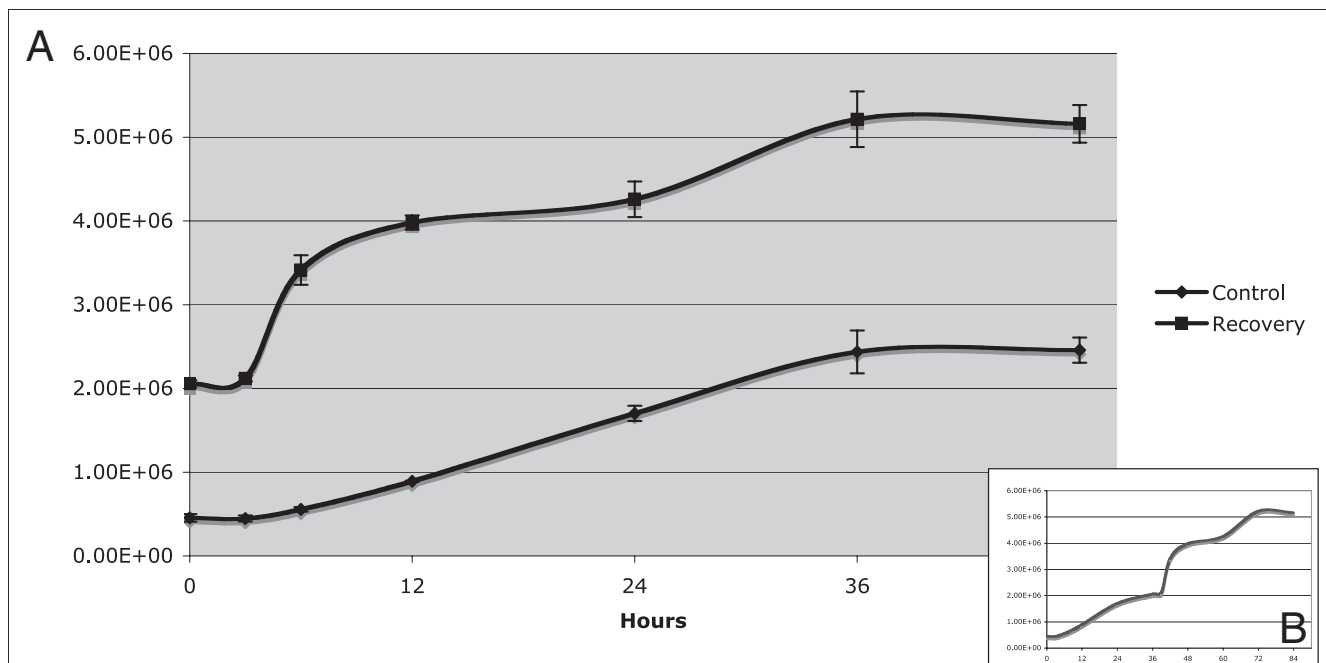


FIG. 4. Growth curves. (A) A growth curve for control cultures starting after the cultures were split (0 h, control curve) and continuing over 48 h was generated. After 36 h of growth, the nocodazole and aphidicolin treatments were performed. Fresh growth medium was added (0 h, recovery curve), and growth was tracked during recovery for an additional 48 h to generate the recovery curve. In panel B, both control and recovery data were graphed in a linear fashion to represent the data as one continuous curve.

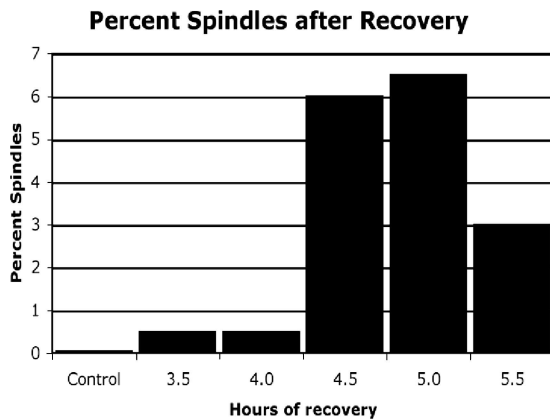


FIG. 6. Percentages of mitotic spindles in *Giardia* cultures following recovery from cell cycle arrest. Light microscopy was used to count tubulin-labeled spindles from approximately 500 cells per *Giardia* culture after 3.5, 4.0, 4.5, 5.0, and 5.5 h of recovery from exposure to nocodazole and aphidicolin treatments. The number of spindles increases after 4.5 h and continues to increase after 5.0 h of recovery but decreases by 5.5 h of recovery, indicating that the wave of mitosis is complete.

due to differences not in the amounts of DNA but in the amounts of fluorescent dye available for incorporation into DNA and should not be considered a disadvantage of our protocol.

## DISCUSSION

In the past, an inability to synchronize *Giardia* cell cultures has made it extremely difficult to study any aspects of cell biology related to the cell cycle. In this work, we developed an efficient method for attaining cell cycle synchrony in a two-step procedure using the drugs nocodazole and aphidicolin. The two-step procedure is necessary for *Giardia* because the parasitic protozoan does not respond as expected to drugs like hydroxyurea and colchicine (7), which are commonly used for cell synchronization for metazoans. This disparity may indicate that the cell cycle of *Giardia* is controlled in a manner different from that for mammalian cells and/or that the giardial targets of these drugs are highly divergent from their metazoan homologs. The use of aphidicolin with *Trypanosoma brucei* (19) previously proved successful as a means of inhibiting nuclear DNA synthesis and initiating cell cycle arrest in the  $G_1/S$  stage (9, 19). However, aphidicolin treatment does not necessarily inhibit cytokinesis, indicating that a mitotic entry checkpoint is activated but that it does not prevent cytokinesis in the absence of mitosis (19). The work presented here provides the first published example of using aphidicolin to trigger cell cycle checkpoints in *Giardia*. Because exposure to aphidicolin causes cell cycle arrest in the  $G_1/S$  stage for both *T. brucei* and *Giardia*, it can be assumed that aphidicolin has the same DNA polymerase target in these early-diverging eukaryotes as in other eukaryotic cells (24). In developing our protocol, we observed that concentrations from 30 to 60  $\mu\text{M}$ , like those used for arrest for *T. brucei* (9, 19), caused cell death for *Giardia*.

Previous studies using nocodazole with *Giardia* investigated the direct and indirect effects of this drug on microtubule destabilization of the cytoskeleton. Exposure to high concen-

trations of nocodazole for prolonged periods of more than one cell cycle resulted in ventral disk fragmentation and abnormally shaped cells and ultimately led to cell death (13). Exposure to 10  $\mu\text{M}$  nocodazole for 5 h had dramatic effects on flagellar length and the size of the median body (6) and on mitotic spindles (20). These results demonstrate that multiple giardial microtubule arrays are sensitive to nocodazole, and it is possible that many (if not all) of them are required for normal cell growth. By lowering the concentration of nocodazole to 100 nM and shortening the exposure time to 2 h, we were able to arrest cells in  $G_2$ . However, upon the removal of the drug, the cells returned almost immediately to their original growth distribution. This result suggests that nocodazole may act by blocking cell cycle progression at multiple points in  $G_2$  or may have slowed cell growth throughout the cell cycle without necessarily triggering the spindle checkpoint, leading to asynchronous cell cycle progression after drug removal.

By combining short incubation times with low concentrations of nocodazole and aphidicolin, we have developed the first cell cycle synchrony protocol for *Giardia* that does not jeopardize cell viability. These data indicate that a  $G_2/M$  boundary checkpoint is presumably activated by depolymerizing spindle microtubules, and upon drug washout, a wave of mitotic activity is observed, resulting in a high number of mitotic spindles compared to those in control samples. The presence of viable spindles during this transition signifies that microtubule organization in the trophozoite has not been irreversibly altered by the nocodazole. Previously, we were able to achieve a lesser degree of cell cycle synchrony by subjecting cells to bile starvation (20). While this method was sufficient to enhance the number of spindles, it may not be suitable for other biochemical studies.

The ability to arrest *Giardia* cultures in  $G_1$  and  $G_2$ , followed by synchronous recovery spanning one to two life cycles, will enhance our ability to study cell cycle-related events. These techniques can be applied to studying not only mitotic spindles but also processes such as the cyclin-dependent kinase control of the cell cycle and the process of encystation.

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