

# Genome ploidy in different stages of the *Giardia lamblia* life cycle

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## Summary

The early diverging eukaryotic parasite *Giardia lamblia* is unusual in that it contains two apparently identical nuclei in the vegetative trophozoite stage. We have determined the nuclear and cellular genome ploidy of *G. lamblia* cells during all stages of the life cycle. During vegetative growth, the nuclei cycle between a diploid (2N) and tetraploid (4N) genome content and the cell, consequently, cycles between 4N and 8N. Stationary phase trophozoites arrest in the G<sub>2</sub> phase with a ploidy of 8N (two nuclei, each with a 4N ploidy). On its way to cyst formation, a G<sub>1</sub> trophozoite goes through two successive rounds of chromosome replication without an intervening cell division event. Fully differentiated cysts contain four nuclei, each with a ploidy of 4N, resulting in a cyst ploidy of 16N. The newly excysted cell, for which we suggest the term 'excystoite', contains four nuclei (cellular ploidy 16N). In a reversal of the events occurring during encystation, the excystoite divides twice to form four trophozoites containing two diploid nuclei each. The formation of multiple cells from a single cyst is likely to be one of the main reasons for the low infectious doses of *G. lamblia*.

## Introduction

*Giardia lamblia* belongs to the earliest diverging eukaryotic lineage known (Sogin *et al.*, 1989). Although it is a true eukaryotic cell, it displays several prokaryotic properties, e.g. lack of mitochondria and peroxisomes, prokaryotic-size small subunit (SSU) rRNA, bacterial-like metabolic enzymes and lack of cap structures in most mRNAs (Sogin *et al.*, 1989; Gillin *et al.*, 1996; Yu *et al.*, 1998). *Giardia* is more divergent

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from yeast, in evolutionary terms, than yeast is from man (Sogin *et al.*, 1989), and elements that are conserved from *Giardia* to man are therefore likely to reflect universal eukaryotic cell functions. Thus, it is a valuable model for gaining basic insights into key pathways that characterize eukaryotic cells and into the early evolution of eukaryotes.

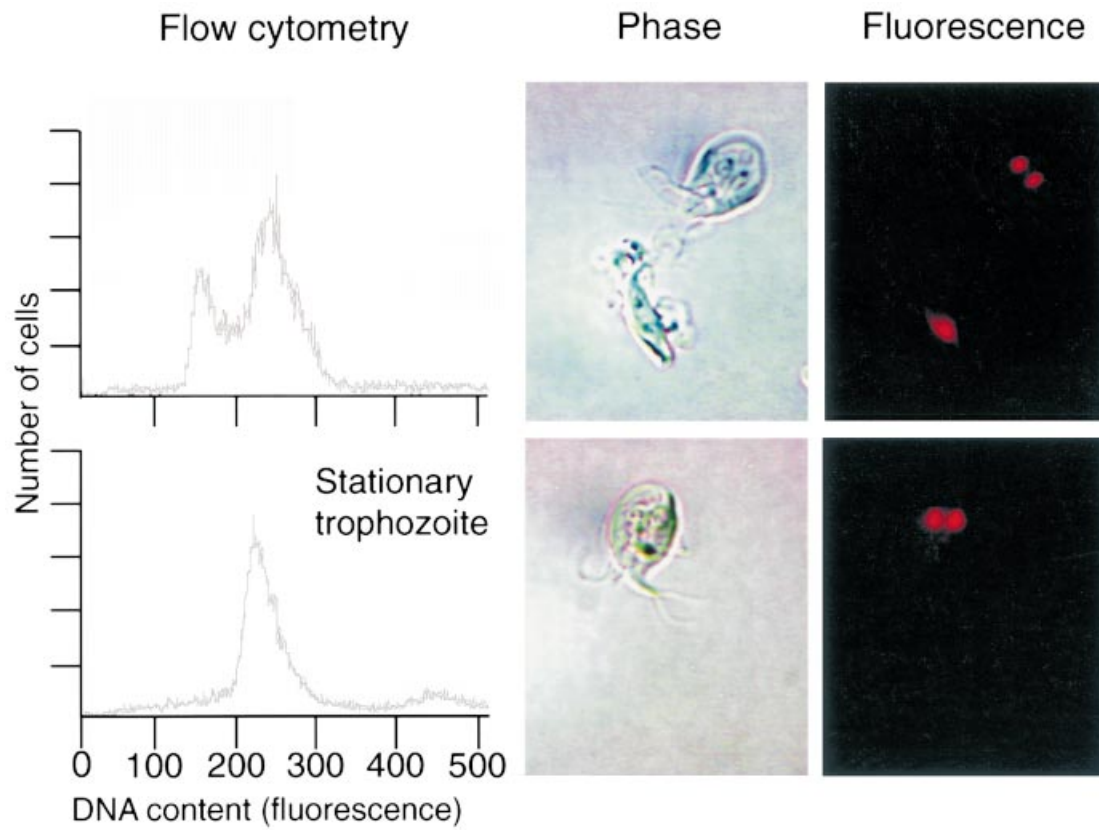
*G. lamblia* is a binucleated protozoan that inhabits the upper small intestine of its vertebrate hosts (Gillin *et al.*, 1996). The entire life cycle can be completed *in vitro* with stimuli that mimic gastrointestinal conditions (Gillin *et al.*, 1996). Infection of the host is initiated by ingestion of cysts, followed by excystation and colonization of the small intestine by the trophozoite form of the parasite, which multiplies by vegetative growth in the intestine. Trophozoites, like most intestinal parasitic protozoa, undergo dramatic biological changes to survive outside the intestine of their host by differentiating into resistant cysts (encystation). Encystation and excystation are among the simplest developmental processes in eukaryotes, and characterization may yield important information about more elaborate developmental processes.

The best characterized *G. lamblia* isolate is WB (ATCC 309571), and the complete genome of WB clone C6 is currently being sequenced (<http://www.mbl.edu/giardia>). Pulse-field analysis of the WB isolate (Le Blancq and Adam, 1998; Adam, 2000) showed that it contains five different chromosomes of approximate sizes 1.6 Mb (chromosomes 1 and 2), 2.3 Mb (chromosome 3), 3.0 Mb (chromosome 4) and 3.8 Mb (chromosome 5), resulting in a haploid genome size of 12 Mb. Recent data show that genome ploidy is important for gene regulation and differentiation in eukaryotic cells (Galitski *et al.*, 1999; Hieter and Griffiths, 1999). The ploidy of *G. lamblia* nuclei and cells has been estimated to be between 2 and 12N (1–6N per nucleus) using different methods (Erlandsen and Rasch, 1994; Le Blancq and Adam, 1998). We have used flow cytometry and phase-fluorescence microscopy to determine the nuclear and cellular genome ploidy during the complete life cycle of *G. lamblia* WB-C6.

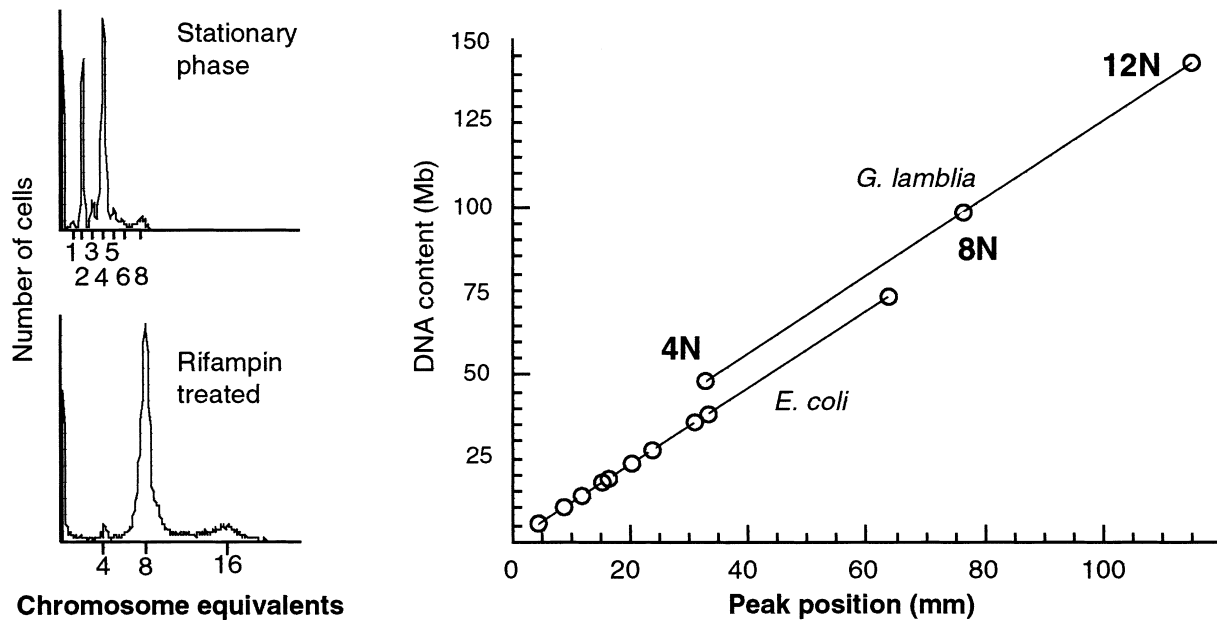
## Results

### *Flow cytometry analysis of vegetatively growing and stationary phase cells*

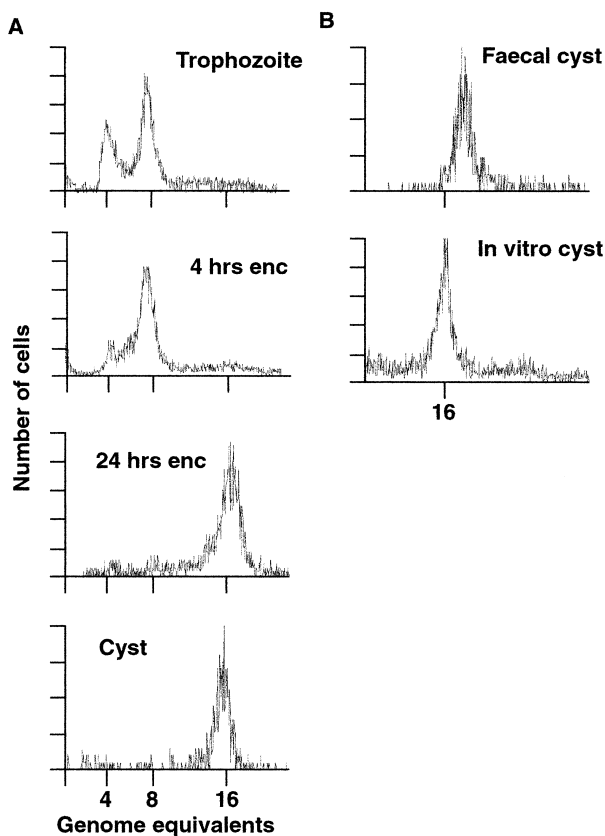
Flow cytometry DNA distributions of exponentially growing *G. lamblia* strain WB, clone C6, trophozoites showed two



**Fig. 1.** Flow cytometry and fluorescence microscopy analysis of the DNA content in vegetative and stationary phase *G. lamblia* trophozoites. Left. DNA content distributions generated by flow cytometry. Middle. Trophozoites visualized by phase contrast microscopy. Right. Epifluorescence microscopy of *G. lamblia* cells labelled with propidium iodide. Note that one of the trophozoites is seen from the side.



**Fig. 2.** Calibration curve for determination of *Giardia* ploidy using *E. coli* strain MG1655 (4.64 Mb) as reference. The 4N and 8N peaks of *G. lamblia* trophozoites are plotted as 48 and 96 Mb, respectively, assuming a haploid genome size of 12 Mb.



**Fig. 3.** Flow cytometry analysis of changes in ploidy during *G. lamblia* encystation and comparison with faecal cysts. A. DNA content distributions of trophozoites before and after induction of *in vitro* encystation. B. DNA content distributions of purified *in vitro* generated and faecal cysts.

major peaks (Fig. 1), representing cells in the pre- ( $G_1$ ) and post-replicative ( $G_2$ , mitosis and cytokinesis) stages of the cell cycle. Fluorescence microscopy combined with image analysis (Malandrin *et al.*, 1999) showed that the nuclei stained with equal brightness, indicating similar DNA contents (Fig. 1). Cells grown to stationary phase displayed one predominant peak, which corresponded to the post-replicative stage (Fig. 1). The peak was slightly displaced compared with the corresponding peak in trophozoite populations, presumably as a consequence of differences in DNA structure between actively growing and resting cells that affect the relative amount of bound stain. A minor peak corresponding to cells with twice the amount of DNA was also detected (Fig. 1). Microscopy analysis of stationary phase cells showed that they were binucleated, demonstrating that the cells had not initiated nuclear division in mitosis (Fig. 1; Cerva and Nohynkova, 1992). Thus, *G. lamblia* trophozoites arrest in the  $G_2$  stage in stationary phase.

#### Determination of DNA content and ploidy

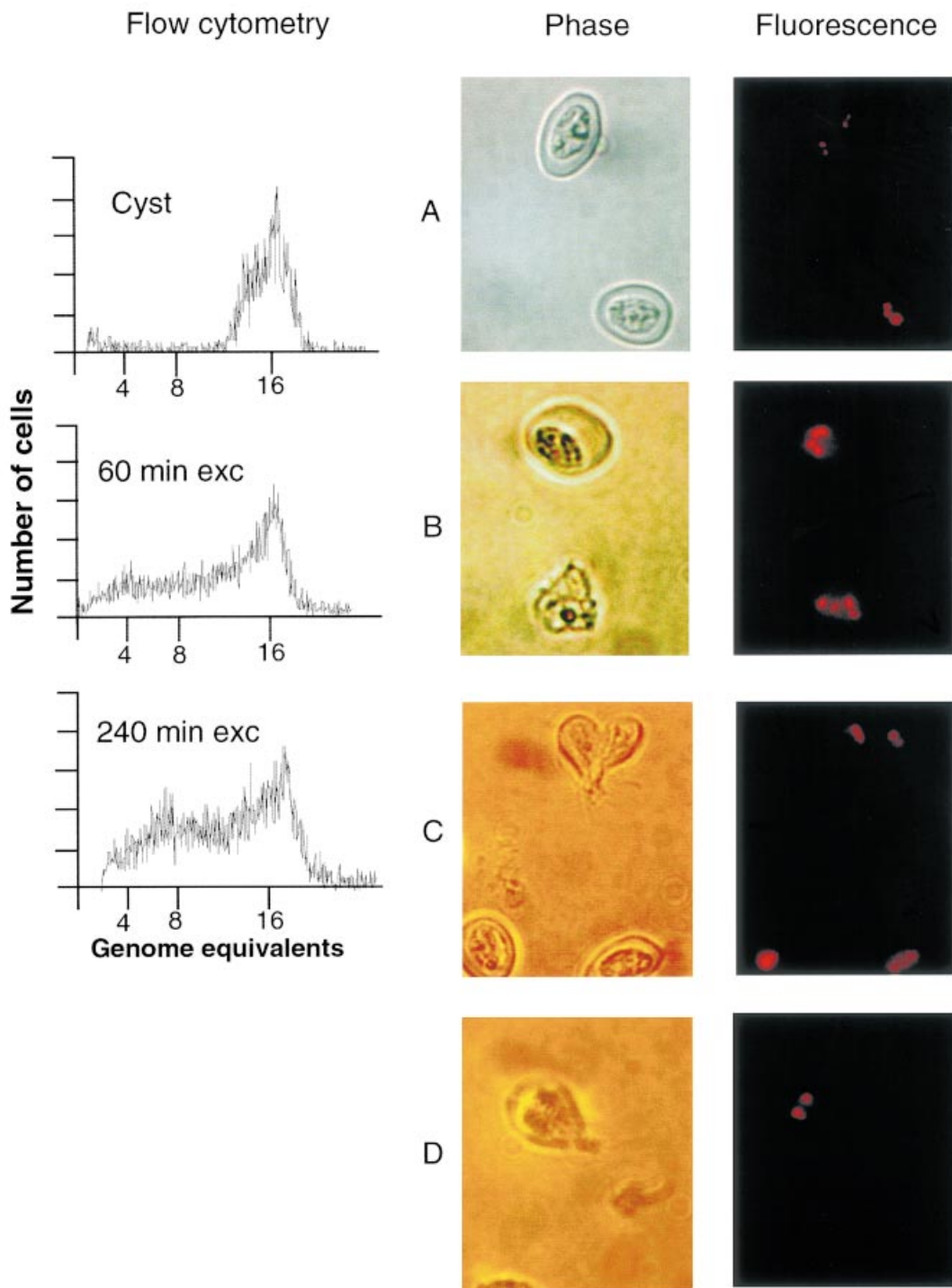
To determine the ploidy, we compared the fluorescence

signals from the trophozoites with those from *Escherichia coli* cells with known DNA content. *E. coli* MG1655 cell populations containing a wide range of fully replicated chromosomes were generated by two different methods. Cultures of MG1655 grown to stationary phase in rich medium contained between one and eight chromosomes (Fig. 2). Also, cultures growing exponentially in rich medium were treated with rifampin, which blocks initiation of chromosome replication while allowing ongoing rounds of replication to continue to termination (replication runout; Skarstad *et al.*, 1996). Cells treated in this way contained four, eight or 16 chromosomes (Fig. 2). Together, this provided a linear DNA scale from 4.64 Mb (a single *E. coli* MG1655 chromosome) to 74.2 Mb (16 chromosomes) (Fig. 2).

The prereplication peak of *G. lamblia* was localized near the 8N peak of *E. coli* and the post-replication peak resided well above the 16N peak. When plotted as multiples of the proposed genome size (12 Mb; Adam, 2000; see *Introduction*), the peaks resided on a line that was perfectly aligned with that of *E. coli* (Fig. 2). This showed that the *G. lamblia* peaks were multiples of a given DNA content, with no evidence of large DNA content variation between the cells. The line was slightly displaced compared with that of *E. coli*, which might be the result of differences in GC content, DNA topology, chromatin organization or other parameters that affect DNA staining. Alternatively, the *G. lamblia* genome size is, in fact, less than 12 Mb; a total genome size of 10.5 Mb would essentially superimpose the two lines and this genome size has been suggested earlier (Fan *et al.*, 1991). With a *G. lamblia* haploid genome size of 10.5–12 Mb, the two peaks could only correspond to 4N (42–48 Mb) and 8N (84–96 Mb) respectively. The 12N point in Fig. 2 is discussed below.

#### Encystation

We wanted to study encystation of *G. lamblia in vitro* and to determine the DNA content of mature cysts. Earlier studies have indicated that cysts contain four nuclei and that the DNA content is twice that of stationary phase trophozoites (Erlandsen and Rasch, 1994). Our demonstration that stationary phase cells are arrested in  $G_2$  with a ploidy of 8N therefore suggests that the ploidy of mature cysts would be 16N. Sixty minutes after induction of encystation, the 4N peak corresponding to newly divided trophozoites decreased, and at 4 h the majority of cells had a ploidy of 8N (Fig. 3A). This is consistent with the observation that trophozoite multiplication was blocked upon induction of encystation (data not shown) and it suggests that *G. lamblia* differentiates from the  $G_2$  stage. After 8 h of encystation, the first mature cysts with four nuclei appeared (data not shown), and by 24 h of



**Fig. 4.** Flow cytometry and fluorescence microscopy analysis of *G. lamblia* excystation.

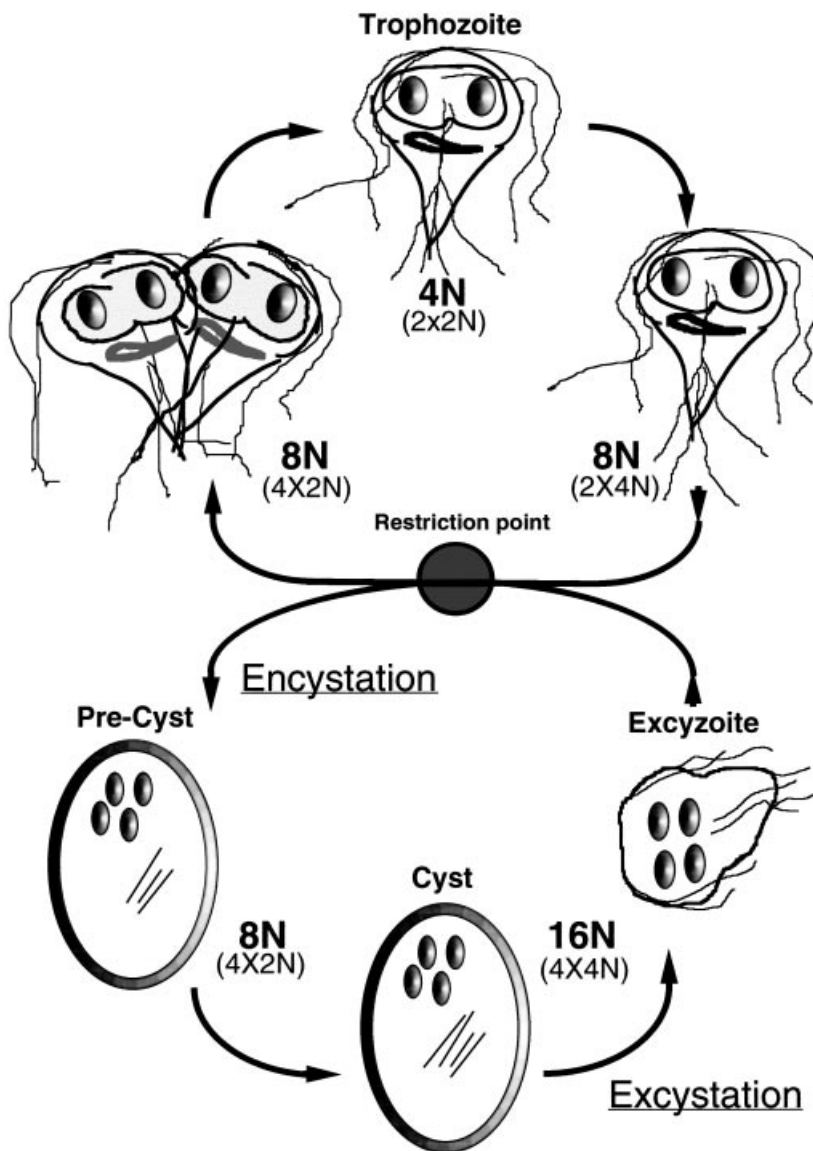
A. Cysts containing four nuclei (16 N).

B–D. Ninety minutes after excystation. Recently excysted cells containing four (B and C) or two nuclei (D) are shown. Left, DNA content distributions generated by flow cytometry; middle, cells in phase-contrast illumination; right, fluorescence signals from labelled cells.

encystation there were mainly quadrinucleate 16N cysts in the population (Fig. 3A).

Current models propose that nuclear division is a prerequisite for DNA replication in higher eukaryotes

(Tada and Blow, 1998). To determine whether nuclear division occurs before DNA replication in encystation, we used fluorescence-activated cell sorting (FACS) analysis of 15 h encysting cells with an equal distribution of cells with a



**Fig. 5.** Proposed model of the *G. lamblia* life cycle. Cellular genome equivalents are indicated in bold, and nuclear ploidy in parentheses. Trophozoites cycle between a 4N and 8N cellular genome ploidy. Upon stimulation of encystation, *Giardia* differentiates from the G<sub>2</sub> stage. The cyst wall is put down and the nuclei divide, resulting in a tetranucleate cell with a ploidy of 8N. Subsequent DNA replication generates mature cysts with a 16N cellular ploidy. Excystation results in release of an excyzoite, which divides to generate two trophozoite-like cells that re-enter the cell cycle in G<sub>2</sub>.

ploidy of 8- and 16N. FACS analysis combined with fluorescence microscopy showed that most cells sorted from the 8N peak had cyst walls and that 70% of these had four nuclei, whereas cells sorted from the 16N peak were cysts with four nuclei (data not shown). Thus, most cells divide their nuclei before DNA replication in encystation.

Purified *in vitro* generated cysts and cysts purified from the faeces of an infected patient with giardiasis also displayed a DNA content of 16N (Fig. 3B). The patient's cysts were fixed differently (see *Experimental procedures*), which might explain the higher fluorescence signals than in the *in vitro* cysts, although small differences in genome size cannot be ruled out. When encystation was suboptimal, low encystation frequencies were observed and the majority of purified cysts had a DNA content of 12N (see Fig. 2). Microscopy analysis

revealed that a majority of these cysts contained three nuclei and that most of these cells were not as symmetrically shaped as those containing four nuclei. We believe that the 12N peak corresponded to immature cysts, in which only one of the two nuclei had undergone S phase and doubled its DNA content.

#### *Excystation*

Earlier observations suggest that, during excystation, the recently excysted quadrinucleate cell quickly divides (within 30 min after excystation) into two trophozoites with two nuclei each (Buchel *et al.*, 1987; Hetsko *et al.*, 1998). Flow cytometry studies of excystation were found to be problematic because excystation frequencies were usually very low, 5–10%, and the recently excysted cells

formed aggregates. Treatment of cysts with acidic reducing solutions mimicking the stomach or basic trypsin-containing solutions mimicking the intestine did not result in detectable ploidy changes (data not shown). After 60 min in growth medium, the main peak was still the 16N peak, but cells with lower ploidy were detected (Fig. 4). Most of the recently excysted cells contained four nuclei (Fig. 4). However, some of the cells with four nuclei were heart shaped and were dividing (Fig. 4), and a few cells with two equally stained nuclei were detected (Fig. 4). The 8N peak increased 4 h after excystation, but the background signal was still high (Fig. 4). Three days after excystation there was a confluent layer of trophozoites in the culture, and a 4- and 8N peak corresponding to trophozoites and a 16N peak corresponding to non-excysted cysts were detected (data not shown). This suggests that the recently excysted cell with a ploidy of 16N ( $4 \times 4N$ ) divided to form two cells with two nuclei and an 8N ( $2 \times 4N$ ) ploidy. These cells subsequently divided into two trophozoites with two nuclei and a cellular ploidy of 4N. Thus, four trophozoites are generated from each cyst.

## Discussion

We have carried out a flow cytometry analysis of the DNA content of *G. lamblia* cells. Our proposed model of the *G. lamblia* life cycle is shown in Fig. 5.

### Ploidy determinations

There are reports of *G. lamblia* strains showing signs of aneuploidy during *in vitro* growth (Upcroft *et al.*, 1996), making these strains less suitable for ploidy analysis. We used the well-defined WB-C6 strain, which contains five equally abundant chromosomes of known sizes. Also, we used flow cytometry, whereas earlier studies of the genome ploidy of *G. lamblia* used densitometry of pulse-field-separated chromosomes,  $C_0t$  analysis or DNA quantification by microspectrophotometry after Feulgen staining (Nash *et al.*, 1985; Fan *et al.*, 1991; Erlandsen and Rasch, 1994). These studies yielded conflicting results (cellular ploidy 2N–12N), at least partly because these methods do not differentiate between cell cycle stages. However, a functional ploidy of four is supported by recent pulse-field data showing four size variants of the five chromosomes in cloned lines of *Giardia* (Le Blancq and Adam, 1998) and by the identification of four different alleles of two different VSP genes (Yang and Adam, 1994).

### The vegetative cell cycle

The two nuclei appear to replicate their DNA synchronously in *G. lamblia* trophozoites (Wieseahn *et al.*, 1984;

Kabnick and Peattie, 1990). Our results show that, during vegetative growth, each nucleus cycles between a diploid and tetraploid genome content, whereas they never appear to go through a haploid stage; a cellular ploidy of 2N was not observed.

### Encystation

Although mammalian cells usually differentiate from the  $G_0/G_1$  stages after cell cycle arrest, some cell types, for example megakaryocytes, differentiate in  $G_2$  (Coffman and Studzinski, 1999). Stationary phase *Acanthamoeba* (a single-celled eukaryote) differentiate into cysts from a proposed restriction point in  $G_2$  (Byers *et al.*, 1991). The encystation frequency is dependent on the number of cells that are arrested in  $G_2$  when encystation is induced. The encysting parasite *Entamoeba invadens* has also been suggested to recognize stimuli for encystation in  $G_2$  (Eichinger, 1997).

Our results indicate that *G. lamblia* differentiates in  $G_2$  in the same way as other encysting eukaryotes. The highest encystation frequencies were observed at 70% confluency, and stationary phase cells had lower encystation frequencies (data not shown). During encystation of *G. lamblia*, two rounds of DNA replication occurred without an intervening cell division event, and our results indicate that nuclear division precedes DNA replication. Endomitosis is a process in which two or more rounds of chromosome replication occur without an intervening cell division and is observed in the differentiation of several cell types, including megakaryocytes and liver, urinary bladder epithelium, trophoblast and salivary gland cells (Varmuza *et al.*, 1988). Further studies will show whether there are conserved mechanisms of cell differentiation and endomitosis in eukaryotic cells.

### The excyzoite

Excystation frequencies of *in vivo* and *in vitro* generated cysts vary between 0% and 50% (Hetsko *et al.*, 1997). A reason for this is probably differences in the percentages of mature, viable cysts in the starting cyst populations. During excystation, the recently excysted cell divides twice without DNA replication. The differentiation in *G. lamblia* is therefore reminiscent of meiosis, in which the genome is first replicated and then divided twice without DNA replication. It is possible that differentiation of primitive eukaryotes into cystic forms is an ancestral form of sexual processes. Meiosis- and spermatogenesis-specific genes have been identified in the *G. lamblia* genome project and their expression patterns will be of interest in relation to these issues.

The recently excysted cell is oval, has eight flagella, four nuclei with a 16N DNA content and a metabolism

intermediate between a trophozoite and a cyst (Paget *et al.*, 1998). We propose that this important life cycle stage, which initiates infection, is designated the excyzoite.

#### *Ploidy and gene regulation*

Many plants and animals generate specific subpopulations of polyploid cells by DNA replication in the absence of cell division during tissue-specific differentiation, and it has recently been shown that changes in ploidy are common in differentiation and very important in gene regulation (Galitski *et al.*, 1999). Similarly, the increase in ploidy to 16N in the excyzoite could induce expression of specific genes and repress others. In support of this, gene expression increases dramatically in the excyzoite (Hetsko *et al.*, 1997).

#### *Infectious dose*

The infectious dose of *G. lamblia* is very low, in the range of 10–100 cysts (Rendtorff, 1954), and may be explained by the fact that each cyst can give rise to four trophozoites. *Entamoeba histolytica* uses a similar approach, such that a multinucleated cyst divides into eight trophozoites (Eichinger, 1997), whereas *Giardia microti*, which infects rodents, divide into two fully developed trophozoites within the cyst (Januschka *et al.*, 1988). An advantage of infecting with a polyploid multinucleated cyst may be that no major biosynthetic investment is required for the production of multiple infectious trophozoites from a single cyst; full commitment to macromolecular synthesis and vegetative growth may thus be postponed until early infection steps have been completed.

### **Experimental procedures**

#### *Reagents, cell culture and differentiation*

Unless otherwise indicated, reagents were obtained from Sigma. *G. lamblia* strain WB (ATCC30957) clone C6 trophozoites were grown vegetatively and encysted as described previously (Kane *et al.*, 1991). Excystation was carried out by a two-step method, as in Boucher and Gillin (1990). Faecal human cysts were purified from fresh faeces and fixed with standard concentration techniques according to Allen and Ridley (1970).

#### *Flow cytometry*

*G. lamblia* cells were fixed for flow cytometry analysis according to Dvorak (1993). Briefly,  $5 \times 10^6$  cells were concentrated by centrifugation at 900 *g*. The pellet was resuspended in 50  $\mu$ l trophozoite culture medium and mixed with 150  $\mu$ l cell fixative (1% Triton X-100, 40 mM citric acid,

20 mM dibasic sodium phosphate and 0.2 M sucrose, pH 3.0). After fixation at room temperature for 5 min, 350  $\mu$ l of diluent buffer (125 mM MgCl<sub>2</sub> in PBS, pH 7.4) was added and the samples were stored at 4°C until use. Importantly, RNase treatment was found to be crucial for obtaining high-resolution DNA content distributions. Fixed cells were concentrated by centrifugation at 5200 *g* and washed once in PBS. Washed cells were resuspended in 500  $\mu$ l PBS containing 2.5  $\mu$ g of RNase (Boehringer) and incubated at 37°C for 30 min. RNase-treated cells were concentrated by centrifugation and resuspended in 65  $\mu$ l of Tris-MgCl<sub>2</sub> solution and 65  $\mu$ l mithramycin A–ethidium bromide solution according to Skarstad *et al.* (1996).

*E. coli* MG1655 cells were grown in Luria–Bertani (LB) medium and prepared for flow cytometry by standard methods (Skarstad *et al.*, 1996). Flow cytometry for DNA quantification was performed on a Brite HS instrument (Bio-Rad) and cell sorting was performed on a FACScan (Becton Dickinson).

#### *Fluorescence microscopy*

Cells were fixed and RNase treated and stained with 0.05 mg ml<sup>-1</sup> propidium iodide in PBS or with mithramycin A–ethidium bromide as described above. Quantification of fluorescence signals from individual nuclei was carried out by image analysis to determine the number of pixels within the area covered by the nucleus (Malandrin *et al.*, 1999).

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