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Studies of encystation-specific DNA replication in *Giardia lamblia*

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Summary

Giardia lamblia is a flagellated protozoon that parasitizes the small intestine of humans and other mammals. The parasite infects more than 250 million humans every year and causes diarrhea. *Giardia* species have two major stages in the life cycle; a vegetative trophozoite and a non-replicating cyst stage. Every step in the *Giardia* life cycle is a response to a stimulus from the host. By using physiomimetic stimuli it is possible to complete the life cycle *in vitro*. When the infectious cyst is exposed to the host's gastric acid the cyst awakens from its cell cycle arrest. The higher pH in the small intestine makes the excyzoite with four tetraploid (4N) nuclei emerge from the cyst. The excyzoite divides twice without any DNA replication. Thus, every ingested cyst produces four tetraploid trophozoites. Encystation is induced by increased pH and high bile concentration. Trophozoites with an 8N ploidy leave the replicative cell cycle and go into dormancy via the encyzoite form. Late in encystation both nuclei are divided and the DNA is replicated, producing a cyst with a cellular ploidy of 16N. Not much is known about DNA replication in *Giardia* and here this process was studied further with a focus on DNA replication in encystation.

A new protocol for encystation of *Giardia in vitro* was developed, lacking starvation in bile-free medium, and found to be superior to the existing protocols when it comes to generation of mature cysts with 16N genome content. This new protocol was used throughout this study. Two inhibitors of the eukaryotic cell cycle (aphidicolin and nocodazole) and two drugs for treatment of giardiasis (flagyl and albendazole) were used on *Giardia* trophozoites and encysting cells *in vitro* to see how they effect DNA replication, cell division and cyst development. Nocodazole interfered with cyst formation and blocked cell division of *Giardia* trophozoites, but the parasite continued to replicate the DNA, which resulted in cells with up to 32N genome content. Albendazole, another tubulin inhibitor used in treatment of human giardiasis, showed similar effects as nocodazole Trophozoites treated with aphidicolin during vegetative growth, at the initiation of encystation and at some point during the first 12 hours of encystations, did not replicate the DNA and no mature 16N cysts were produced. Flagyl, another drug used to treat human giardiasis, did not affect DNA replication and encystation

To see how DNA replication is regulated in *Giardia*, analyses to identify an origin of replication were made with *Giardia* microarrays and marker frequency analysis. In this study these whole-genome arrays were used in an attempt to identify the organization, structure and localization of different replication origins relative to various genes present on the array, i.e markers. In the present study no specific origins of replication could be identified. This is in line with results from higher eukaryotes and one explanation that has been suggested is that eukaryotic cells are replicated from several origins with flexibility in origin selection and firing.

1. Introduction

1.1 Cell Biology of *Giardia lamblia*

Giardia lamblia has different forms during its lifecycle. The vegetative forms, trophozoites, are pear-shaped, 12-15 μm long and 5-9 μm wide, and live inside the small intestine of the host. The dormant form, the infectious cyst, is formed in the lower part of the intestinal system but it spends most time outside the host. The trophozoites have two nuclei that are nearly identical but without nucleoli. The giardial cytoplasm contains lysosomal vacuoles, cytoskeletal organelles, ribosomal and glycogen granules (Adam 2001).

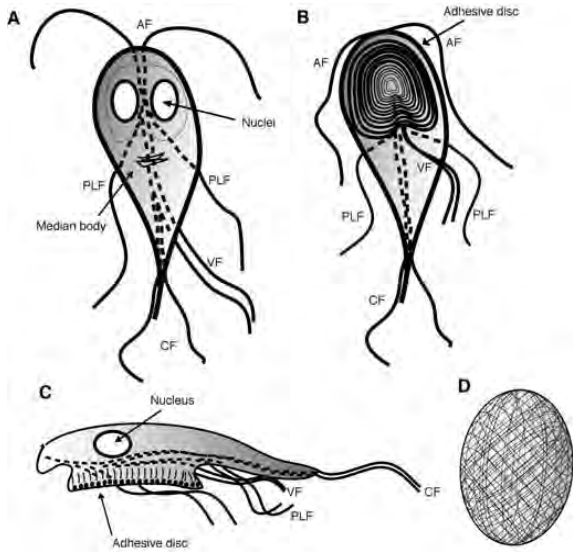

Fig. 1; *Giardia* trophozoites. A) Dorsal view, showing the two nuclei and the median body, B) ventral view, giving a clear image of the adhesive disc, and the four pairs of flagella, C) side view of the parasite, D) Giardial cyst. (Image: S. Svärd, Uppsala Universitet)

2001). The nuclei divide almost at the same time and have the same amount of DNA. The current view is that the two nuclei have the same set of genes and chromosomes (Morrison *et al.* 2007). The ventral disc and the cytoskeleton play important roles for the survival of *Giardia* in the host. *Giardia* colonises the wall of the small intestine and to attach at the epithelial cells in the small intestine *Giardia* has a ventral adhesive disc, which can be seen in Fig. 1 above. The disc makes it possible for the parasite to obtain nutrients and to avoid being transferred further down than the jejunum in the intestinal system (Adam 2001). The ventral disc is an exceptional construction and a component of the cytoskeleton (Adam 2001). The disc has a concave structure and upon attachment it becomes even more concave (Elmendorf *et al.* 2002). *Giardia* has evolved a different type of protein sorting and transport machinery. One example of this is the encystation specific vesicles (ESVs), which are in charge of transporting and sorting newly synthesized cyst wall proteins and glycans during the encystation process (Hehl & Marti. 2004). The growth rate of the *Giardia* WB-C6-A11 lineage, used in this study, appears to be the fastest out of all known, *in vitro* cultured, giardial isolates; about 6 hours (Reiner *et al.* 2008).

1.2 DNA replication in *Giardia*

Giardia contains two approximately identical and synchronously replicating nuclei in trophozoites (Bernander *et al.* 2001). Analyses of the *Giardia* WB isolate showed that the haploid genome size is around 12 Mb and that it contains five chromosomes (Svard *et al.* 2003). Both the cellular and the nuclear ploidies have been determined throughout the *Giardia* life cycle (Bernander *et al.* 2001). During vegetative growth each nucleus cycles between a diploid (2N) and a tetraploid (4N) genome, giving a cellular ploidy of 4N and 8N

(Bernander *et al.* 2001; Svard *et al.* 2003). There are two essential parts of the vegetative cycle; genome replication that takes place during the S-phase and the equal division of the chromosomes to each one of the daughter cells in the M-phase (Svard *et al.* 2003; Adam 2001) . Replication of the DNA also has a highly important role during the encystation process (Svard *et al.* 2003). Only a few of the genes that are known to be important in the regulation of DNA replication in other eukaryotes have been identified in *Giardia* (Morrison *et al.* 2007). After induction of the encystation process, trophozoite multiplication is blocked and the number of 4N cells decreases at the same time as the 8N population increases. It has been shown that the restriction point for encystation, where the trophozoites differentiate into cyst, is located in the early G2 phase of the cell cycle (Svard *et al.* 2003; Reiner *et al.* 2008). Differentiation, i.e. encystation and excystation, in many ways looks like meiosis, where the genome is first replicated without division and then divided twice without DNA replication. As a result of that, S phase and M phase are not coupled to each other in encystation and exystation, which is also the case in meiosis.

1.3 The *Giardia* life cycle

The *Giardia lamblia* lifecycle consists of two major stages: the vegetative trophozoite stage, which is the form that inhabits the upper part of the small intestine of its vertebrate host and the dormant cyst stage that spends most of the time outside the host (Lauwaet *et al.* 2007). An infection starts with ingestion of the infectious cysts, which is followed by an excystation. Excystation means that the cysts transform into trophozoites. Trophozoites divide by binary fission, meaning that the parasite undergoes asexual reproduction, and colonizes the small intestine (Roxstrom-Lindquist *et al.* 2006). When the parasite is exposed to the host's gastric acid a signal induces the dormant cysts to awaken from cell cycle arrest. The pH in the small intestine makes the quadrinucleate daughter cell, the 16 N excyzoit, emerge. Every ingested cyst produces four trophozoites. In contrast to the excystation process, encystation is induced by increased pH and bile.

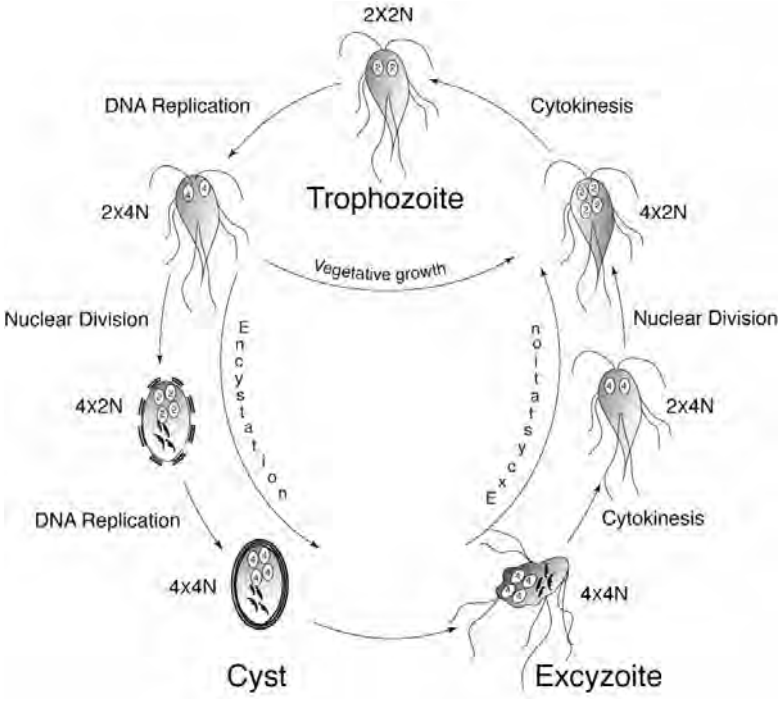

Fig. 2; The *Giardia lamblia* lifecycle. Differentiation from trophozoite to cyst (encystation) and from cyst to trophozoite via the excyzoite (excystation). (Image: D. Palm, SMI, KI, Solna)

The cell goes from the cell cycle to dormancy via the encyzoite form. Late in encystation both the nuclei are divided and the DNA is replicated, producing a cyst with a cellular ploidy of 16 N (Reiner *et al.* in press).The encystation process has two major phases; the early phase and the late phase. In the early phase the intracellular synthesis and transport of cyst wall components takes place, and a Golgi-like stack of membranes becomes visible (Adam 2001; Svard *et al.* 2003). These have been suggested to be a part of the transport machinery of cyst-specific molecules to the encystation secretory vesicles (ESVs). When encystation is initiated the motility of the parasite disappears. The outer segment becomes rounded and the parasite looses its ability to attach to the surface. The late phase of encystation consists of gathering of the filamentous segments into a complete cyst wall. The ESVs disappear and the internal part includes one trophozoites with four nuclei (tetraploid) that has not yet completed cytokinesis (Adam 2001). After exposure to a suitable environment, excystation takes about 10 minutes and the excyzoite immediately commences cytokinesis once it is released into the small intestine (Adam 2001; Bernander *et al.* 2001).

1.4 Pathogenesis

The infection starts with ingestion of *Giardia* cysts of the host. *Giardia* cysts initiate the excystation process, reforms to trophozoites and start to colonize the small intestine. The trophozoites attach to the epithelial cells of the mucosa in the small intestine. They do not invade or attack the epithelial cells mechanically or by secretion of lytic substances (Kreier 1995).

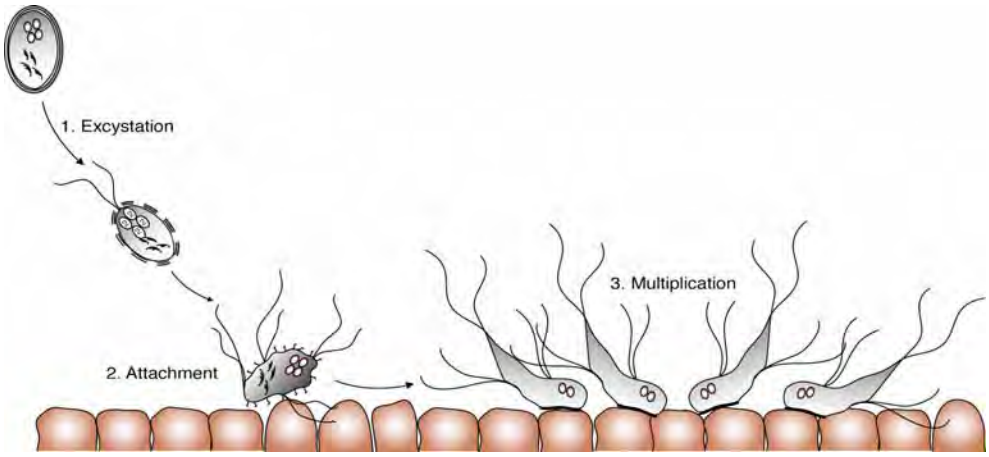

Fig. 3; Schematic picture of the *Giardia* infection. Excystation and colonization of the proximal small intestine. 1) Excystation, triggered by differentiation of the surrounding environment in the gut and the intestine of the host. 2) Excyzoite attachment to the intestinal lumen. 3) Multiplication and colonization of trophozoites in the small intestine. (Image: D. Palm, SMI, KI, Solna)

The disease is characterized by malabsorption, diarrhea, and growth failure in children and it has recently been included in the ”Neglected Diseases Initiative” of WHO (Savioli *et al.* 2006). The sources of disease or models by which the parasite causes injury are still not well known. No virulence factors have been identified yet (Kreier 1995).

1.5 Parasite-host interactions

The optimal life-style of an intestinal parasite is to infect the host, colonize the small intestine and replicate heavily without causing too much damage to the host. Seen from an evolutionary perspective, parasites must avoid extinction by multiplying in their hosts, and then spreading out to the environment and further to other hosts. If the infection is a chronic

infection it is often less problematic and the symptoms are often weak or non-existing. The parasites can more or less blossom in the hosts intestine without getting any major problem with the immune system of the host (Ankarklev 2006). There are natural barriers that are supposed to confront the parasite such as the mucosal layer on the epithelial cells and high concentrations of bile and digestive enzymes. The epithelial cells are constantly turned-over, which results in regeneration of the epithelial surface every three to five days. This makes it essential for the *Giardia* trophozoites to reattach to avoid elimination by the peristalsis (Roxstrom-Lindquist *et al.* 2006). All the proteases, lipids and bile kill almost every other microbe in the upper small intestine, while *Giardia* seems to be protected.

1.6 Immune responses in the host

There are two major subgroups in the human immune system. The adaptive immune system involves response to the challenge with high degree of specificity as well as "memory"; cell-mediated and humoral immune responses. The cell mediated response involves T-cells, also called T lymphocytes. There are two different types of T cells, the CD8+ T-cells and the CD4+ T-helper cells (Goldsby *et al.* 2003). Humoral immunity is mediated by (Abs) antibodies, produced by B cells or B lymphocytes that interact with the antigens. The Abs are present in the serum and in mucosal secretions (Goldsby *et al.* 2003). Innate immunity involves defence mechanisms of a biochemical and cellular nature that are present always and ready to react immediately upon any infection (Goldsby *et al.* 2003). The innate immunity is composed of four groups of defence barriers, 1) cytokines, which are proteins that systematize and regulate many of the cellular activities involved in this type of immune defence; 2) plasma proteins, which are mediators of inflammation, such as the complement system; 3) phagocytic cells, i.e. natural killer cells, neutrophils and macrophages; and 4) chemical and physical barriers, such as epithelia and substances of anti-microbial nature produced at the epithelial surfaces (Abbas 2003; Goldsby *et al.* 2003). Immunological and non-immunological factors have been shown to have different important roles during infection of *Giardia*. Immunity against *Giardia* has been shown to occur in two phases in a study on mice. After the first two weeks of a *Giardia* infection there is a B-cell independent phase which is followed by an antibody-dependent phase. There are clinical reports that show that human giardiasis is correlated to injury of digestive and abortive functions of the intestinal cells (Roxstrom-Lindquist *et al.* 2006). The inflammatory mechanisms like neutrophils, which have been shown to be important against pathogens such as *Salmonella,* are not shown to be important in anti-giardial host defence (Eckmann 2003). The production and delivery of effector molecules against *Giardia* infection is likely to be regulated by a network of different cells, which are not directly involved in the killing of the parasites. In a study of mice that lack specific populations of cells due to genetic deficiency one could see that CD4 αβ T cells were required for clearing the *Giardia* infection while the CD8 T cells and natural killer cells had a passive role or no role at all (Eckmann 2003).

1.7 Parasite evasion of the host immune defence

Giardiasis is self-limiting in > 85% of cases, which indicates that effective host immune defences exist (Eckmann 2003). Chronic cases are a problem in patients with evident immunodeficiency (Kreier 1995).The host inflammatory response is not significant for the disease pathogenesis and the diarrhoea in the vast majority of cases. Nitric oxide (NO) is one substance that is produced from the epithelial cells as a defence against the parasite attack. NO is produced enzymatically from arginine through the action of NO synthetases (NOS). Results from *in vitro* studies of co-culturing giardial trophozoites with human intestinal cells have indicated that trophozoites have the ability to suppress epithelial NO production, not by interfering with the inducible nitric oxide synthetase (iNOS) expression, but rather due to the

high efficiency in consuming arginine. Arginine is an important energy source for the parasite. *Giardia* has developed an arginine transporter system, that has a substrate affinity comparable to that of the human arginine transporter but with a 10 to 20-fold higher maximal transport capacity (Eckmann 2003). Another important way in combating the host immunological force is the presence of variant-specific surface proteins. These proteins are described in greater detail below.

1.8 Variant-specific surface proteins (VSP s)

Giardia lamblia has surface proteins called variant-specific surface proteins (VSPs). VSPs are a family of linked proteins that coat the entire surface of the parasite, even the flagella**.** VSP function as a natural defence against attacks from the immune system. During excystation the parasite undergoes surface antigenic variation (Nash 2002). Most typical is that only one VSP is expressed at a time in one parasite. Dual VSP expression on trophozoites is rare but it occurs when the VSPs are in the process of switching (Nash *et al.* 2001). When a special VSP has been expressed on the parasite and recognised by the immune system of the host, the parasites that express that particular VSP will be killed. This allows for repopulation of giardial trophozoites that express other VSPs (Nash 2002).

1.9 Cell cycle inhibitors

Numerous methods have been used to inhibit cells at some point in the cell cycle and thereby synchronize the cell population. This can be done with starvation methods and by the use of different inhibitory agents. Below are described four different drugs that can be used for this purpose.

1.9.1 Aphidicolin

Aphidicolin is a tetracyclic diterpene antibiotic with antiviral and anti-mitotic properties (Bucknall *et al.* 1973). It is a mykotoxin produced by fungi such as *Nigrospora* sp. (Makioka *et al.* 1998). Aphidicolin is a reversible inhibitor of eukaryotic nuclear DNA replication that acts by inhibiting the replicative DNA polymerase. It blocks the cell cycle at early S-phase (Makioka *et al.* 1998).

1.9.2 Nocodazole

Nocodazole is an anti-neoplastic agent that has the ability to interact with the tubulin and disrupt microtubules (Samson *et al.* 1979). Cells treated with nocodazole were arrested with a G2-phase amount of DNA when analysed by flow cytometry (Cooper *et al.* 2006). Microtubules are 25 nm fibre formed from heterodimers of alpha and beta- tubulin, and are one of the components of the cytoskeleton. The microtubule network has several important roles in the cell, such as cell motility, vesicular transport, formation of the mitotic spindle and in cytokinesis (Elmendorf *et al.* 2002).

1.9.3 Albendazole

Albendazole is known as a tubulin inhibitor. Albendazole disrupts the microtubules of the *Giardia* ventral disc, which makes it hard for the parasite to attach. It has also been proposed that albendazole acts at the nuclear level. The mitotic spindle, which is a microtubule organelle, is essential for nuclear division. In *Giardia* the basal bodies also play an important role in cell division. It is therefore possible that albendazole interferes with the spindle formation and as a result of that, suppresses growth and differentiation of the cells (Sandhu *et al.* 2004).

1.9.4 Flagyl

Flagyl, or metronidazol, is a medical compound that has an inhibitory effect on anaerobic bacteria and certain protozoa such as *Entamoeba*, *Giardia* and *Trichomonas*. Metronidazol is the same thing as Flagyl®, Metronidazol and Rozex® . The compound has an anti-giardial effect both *in vitro* and *in vivo*, and is routinely used for the treatment of giardiasis. However, it has been shown that this drug has no effect on the cysts (Harris *et al.* 2001).

1.10 Flow cytometer

Samples were analyzed by an Apogee A40 flow cytometer. The flow cytometry apparatus works in this way. Cells flow through a flow chamber. A laser hits the cells as the pass through. The way the light bounces off each cell gives information about the cell's physical characteristics. The light goes in to a detector that processes the light and sends the signals to a computer. Then there are filters that direct the light emitted by the fluorochromes to the colour detectors. The fluorochromes that have attached to the cells absorb light as they pass through the laser, and emit a specific colour of light depending on type fluorochrome. The colour detectors collect the different colours of light emitted by the fluorochromes. The fluorochrome data signals are sent to the computer. Data from both the light- and color detector is sent to a computer and plotted on a graph, called histogram.

1.11 Marker frequency

Marker frequency analysis is a method based on the fact that the number of copies of chromosomal DNA markers closely located to a replication origin is higher than the number located near the terminus in a replicating cell population. Marker frequency is a microarray based approach that provides a powerful tool to study the initiation of replication at a genome-wide level. The recently published genome of *Giardia* (Morrison *et al.* 2007) has enabled the production of whole-genome DNA microarrays for this organism. A DNA microarray is a collection of microscopic DNA spots, arrayed on a solid surface by covalent attachment to a chemical matrix. In marker frequency analysis genomic DNA from one sample with replicating cells is hybridized to the same array as a sample where all cells have completed the replication. In this study these whole-genome arrays were used in an attempt to identify the organization, structure and localization of different replication origins relative to various genes present on the array, i.e markers.

1.12 Aim of the study

The aim of the present study was to try different models for encysting cells *in vitro* with different media, and to evaluate which encystation method was the most optimal. With this encysting system a range of encystation experiments were set up to study the DNA replication during differentiation in the presence of different drugs. The studies were performed by cell culturing followed by analyses by flow cytometry and fluorescense microscopy. Another aim was to look for origins of replication in replicating *Giardia* cells with marker frequency and microarray analyses.

2. Results

2.1 Flow cytometric assessment of cells that have grown in different kinds of medium

There are several different ways to encyst *Giardia in vitro.* To induce encystation *in vitro* different environments can be achieved by using different media. Here I evaluated the media conditions for encystation (fig 4). All cells started in the regular tryptone, yeast, Diamond and Keister medium (TYDK medium) containing bovine bile, and were grow to full confluence. In the first protocol the bile was removed for 24 hours to starve the cells from bile, and cells later suspended in to tryptone, yeast, encystation medium (TYE medium) containing porcine bile. This is the widely used protocol for *Giardia* encystation. The second protocol tested was the procedure used in the encystation experiments in this report, which is a method similar to the first, but without starvation of the cells. The water treated cells were cells that after the encystation were treated with water for 24 hours where all cells but mature cysts were lysed (fig.4). The third protocol was like the second but here I tested how a double bovine bile concentration would affect the speed and the efficacy of the process.

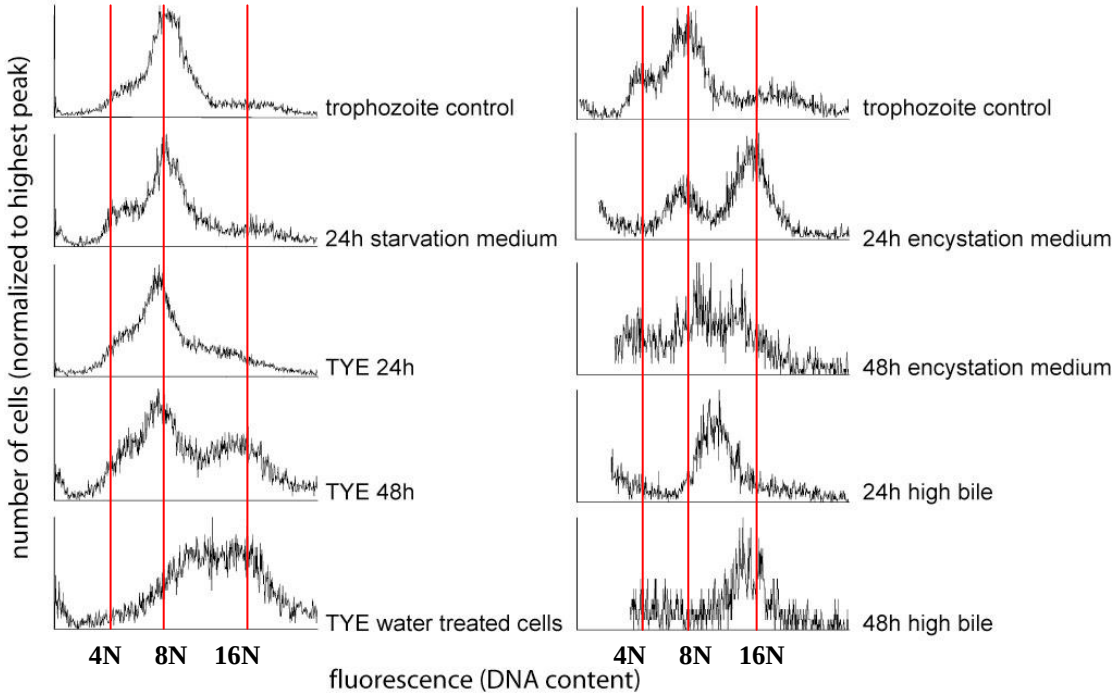

Fig. 4; Assessment of different encystation medium. The left columns show trophozoite control and cells that have grown in starvation medium for 24 hours and cells that have encysted in TYDK-medium for 24 and 48 hours, and one sample that was water treated, to get ride of all remaining trophozoites. The right column shows trophozoite control and cells that had encysted in encysting medium and in encysting medium with double bile for 24 and 48 hours.

As the most widely used protocol runs for 48 hours in encystation medium all three protocols were performed for both 24 and 48 hours for comparison. However, the results showed that 48 hours were not suitable for encystation with the two later protocols. The first protocol was quite slow, and there were still many cells at 4N after 48 hours. The water treatment of the cysts also showed that the cysts produced were not all 16N cysts, which is a requirement for viable cysts. Many cysts with 12N genome content were formed. In this particular experiment something went wrong with the encystation medium with high bile concentration which made it very difficult to fix the cells and very few cysts were produced. Since the results showed that the medium that I called encysting medium (without starvation) gave most mature 16N cyst after 24 hours of encystation, I chose that for further experiments.

2.2 Flow cytometric assessment of encysting cells

To identify the point of DNA replication during encystations a time- course experiment was performed. The cells were transferred to encystation medium and were collected after different times, fixed and analyzed by flow cytometry (fig. 5). After 12-15 hours in encystation medium some of the trophozoites had started to form mature 16N cysts. Most cells had replicated their DNA from 8N to 16N after 21 hours, and after 24 hours about 40-45% of the cells had formed mature cyst.

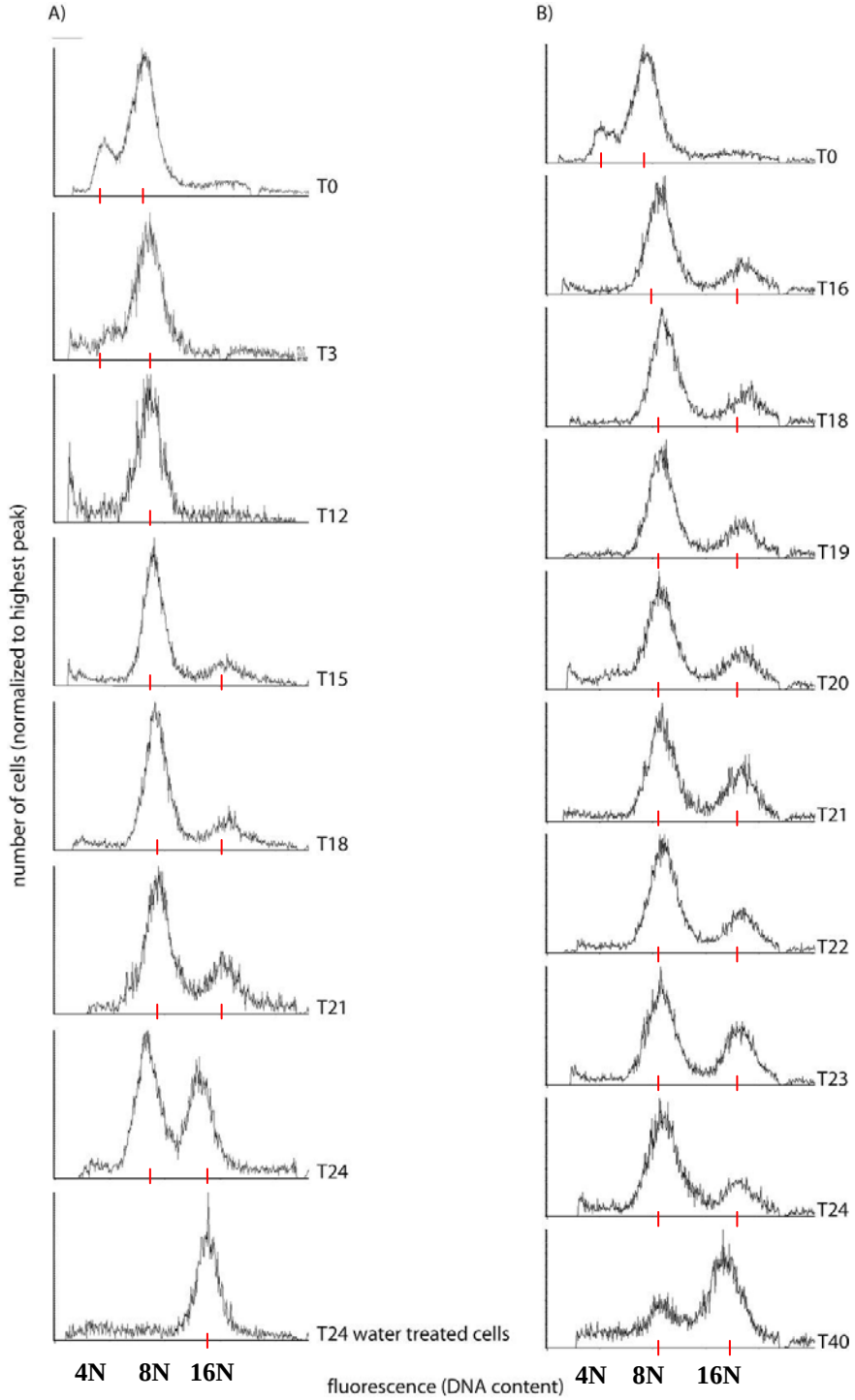

Fig. 5; Cells encysted for different times. Different of tubes with trophozoites cultures were grown in TYDK medium to full confluence. Then medium was changed to encysting medium in all samples but the control (T0). The remaining samples were encysted and fixed at different time points. The first peak illustrates cells in the G1 phase (4N), the second peak illustrate G2 phase cells (8N). A) Totally 8 samples were grown and isolated between 0 and 24 hours. One sample, 24h after change of medium was water treated to get ride of all the remaining trophozoites. B) Totally 10 samples were grown and isolated between 0 and 40 hours.

The last sample after 24 hours was water treated, see fig. 5A. In a second encystation experiment performed as the previous, the exact time of replication was further studied (fig 5B). Both experiments showed that the first mature cysts were seen after 15-16 hours, and that after about 20 hours of encystation (late phase of encystation) most of the cells were on the way to replicate from 8N to 16N.

2.3 Flow cytometric assessment of encysting cells treated with different drugs

Different drugs can be used to influence different stages of the cell cycle. Here, four drugs were tested to asses their effect on the DNA content in encysting cells. Cells were cultivated and encysted the same way as described above, but this time they were also treated with drugs. Altogether four different drugs known to affect the cell cycle were tested (fig 6). The sample with aphidicolin showed no mature 16N cysts after 24h compared to the control. In contrast, the samples with albendazole and nocodazole showed that a majority of the cells had replicated the DNA. Both with albendazole and especially with nocodazole there was an additional peak at 32N which indicated over-replication. Flagyl appeared to have no effect at all on the replication during encystation as the graph looked like the encysting control.

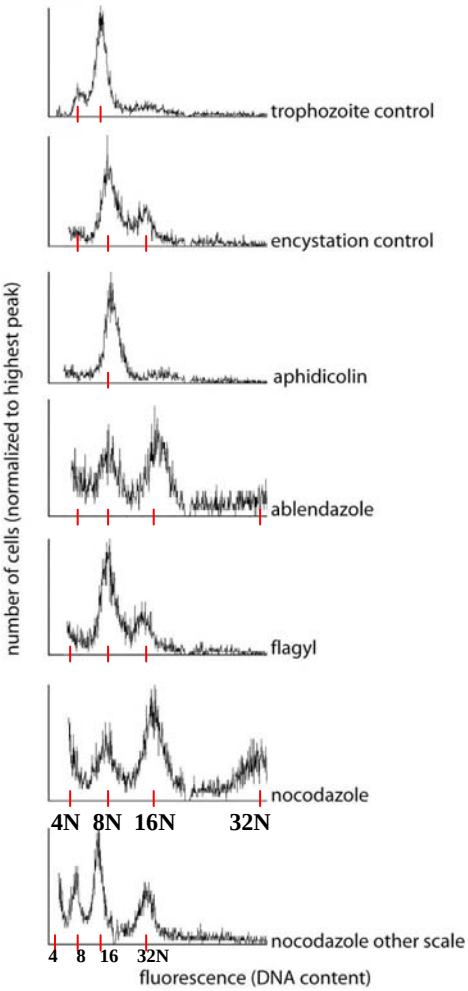

Fig. 6; Encysting cells treated with different dugs. Six samples of trophozoites were grown in TYDK medium to full confluence. Then the medium was changed to encystation medium in 5 samples, and at the same time 4 different drugs were added in 4 of the 5 samples, one drug in each sample.The drugs were aphidicolin, albendazole, flagyl and nocodazole. Untreated trophozoites show a normal population of growing trophozoites. The encysting control shows cells that have encysted in encystation medium for 24h.

2.4 Flow cytometric assessment of encysting cells treated with nocodazole and aphidicolin at different time periods

From the four tested drugs I decided to continue working with nocodazole and aphidicolin as those drugs have different inhibitory actions and dramatic affect on the cells. Cells were cultivated to form cysts and the drugs were added at different times during the encystation process. The first addition of drug was after 3 hours. One sample acted as a control, where no encystation medium or drug was added. One sample was used as an encysting control, i.e without any drug, results are shown in fig. 7. Samples with aphidicolin showed that the drug inhibited the replication to 16N. However, after 15 hours aphidicolin seemed to loose its ability to block the DNA replication as mature 16N cysts had been formed. Samples with nocodazole showed a small 32N peak and a large16N peak after 3 hours of encystation. This peak was remarkably smaller after 9 hours of encystation. At 9 hours and the time points after, the drug did not seem to have any effect of the amount of DNA in the encysting cells.

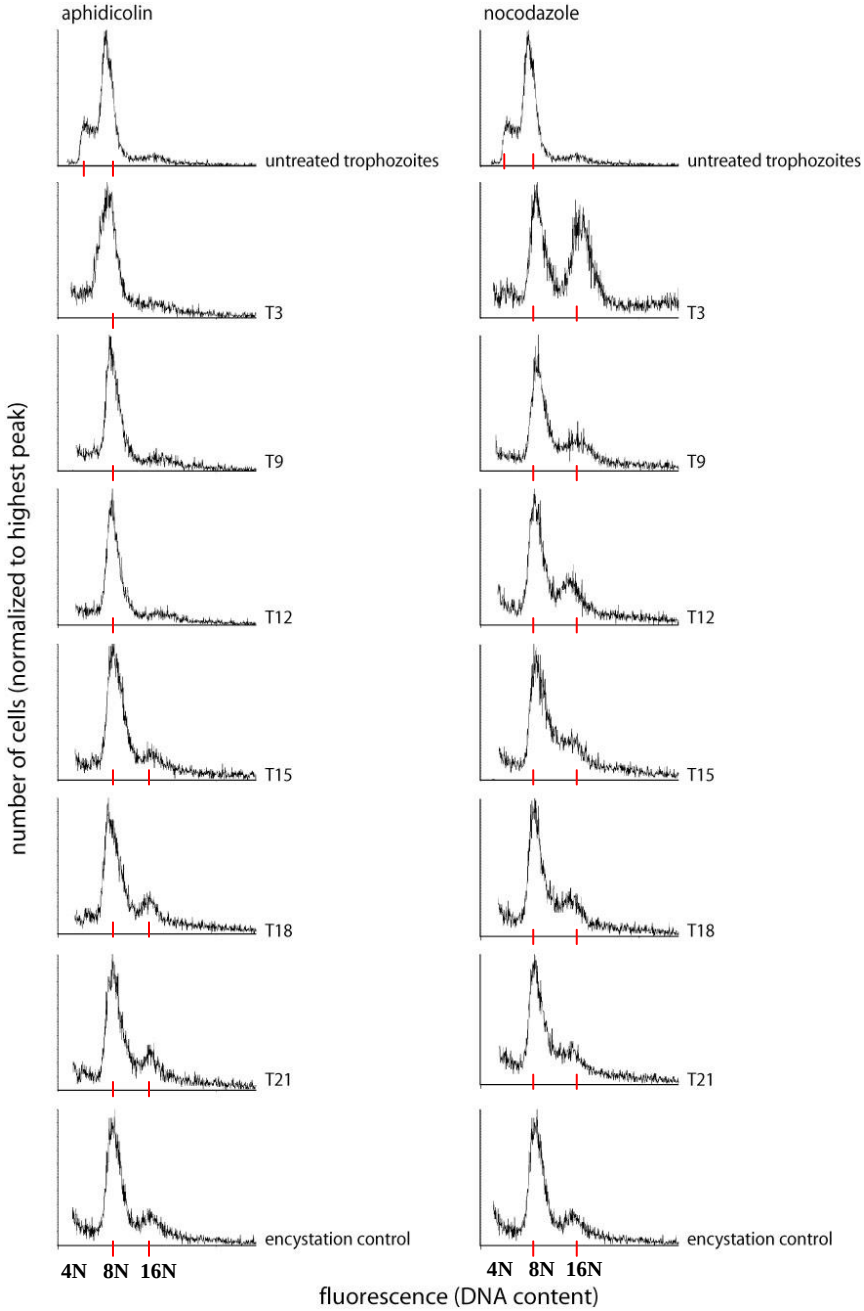

Fig. 7;Time studies on encysting cells grown with nocodazole or aphidicolin. Nocodazole and aphidicolin were added at different time points after initiation of the encystation.

2.5 Flow cytometric assessment of cells treated with aphidicolin or nocodazole either during cell growth and/or during encystation

To see where the different drugs had the largest effects, they were added at different stages in the developmental process from trophozoite to cyst. Cells were grown in TYDK to full confluence. One sample acted as a control, where no encystation medium or drug was added (fig 8, -). One sample was used as an encysting control i.e without any drug in either the growth medium or encystations medium (fig 8, --). One sample was treated with apidicolin only during vegetative growth and not in the encystation medium (fig 8, +) Most cells were inhibited at 4N and approximately 15 % had 8N. The cells treated with aphidicolin in the encystation medium (fig 8, - +) were arrested at 8N, while the cells treated the same way with nocodazole looked as the control but with a much lager 16N peak. This might be due to trophozoites that replicated from G2 without cell division. In the samples where the drug had been added during growth before start of encystation about 1/3 of the aphidicolin treated cells had encysted, whereas very few cells encysted in presence of nocodazole.

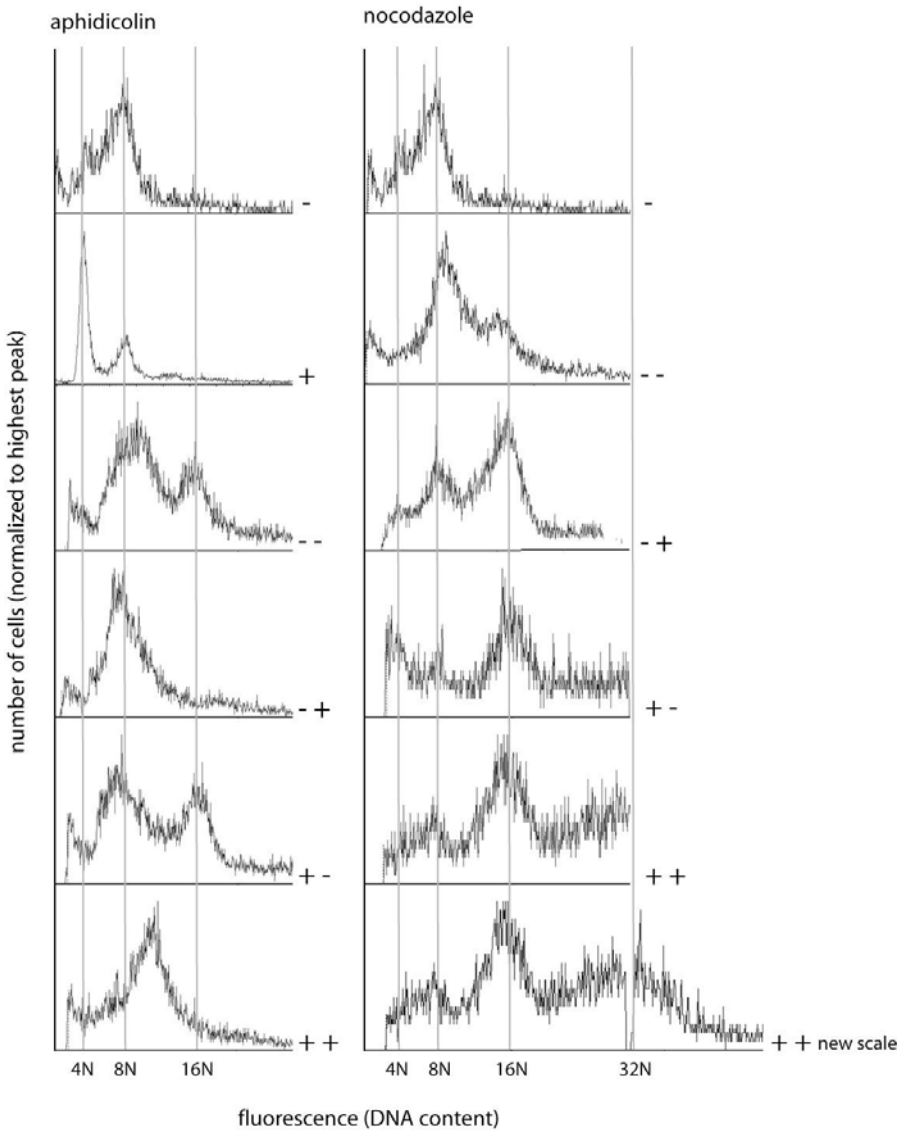

Fig. 8; Cells grown with or without aphidicolin or nocodazole. Cells were grown with or without aphidicolin or nocodazole in TYDK medium and/or in the encysting medium. + + means that drug was added in both the growing- and encysting medium. + - indicate cells grown in TYDK medium with drug and encysting medium without drug. The opposite for - + whereas - + indicates that the drug was added only in the encystations medium. No drug added; - - worked as a encystation control. The sample with only + was treated with apidicolin during growth and no encystation medium was added (this sample was run at a different time than all other samples)

All cells had time to complete one cell cycle before addition of the encystation medium. This means that the trophozoites had time to replicate from G2 without division. The same result was seen in cells treated with nocodazole during both growth and encystation (see + + in fig. 8). Cells treated with aphidicolin during both growth and encystation showed a peak with cells that were 8-12N. The cells were treated for a very long time with aphidicolin, which inhibited the cells, but after while they had escaped inhibition to some extent. This has been observed earlier in cells treated with aphidicolin during vegetative growth (Reiner *et al.*2008).

2.6 Marker frequency analysis of trophozoites to identify origins of replication

No replication origins have been identified so far in *Giardia* trophozoites. In order to study this I performed two marker frequency experiments. I enriched for replicating *Giardia* trophozoites in two different ways. The first was a block with aphidicolin to arrest at G1/S, followed by release for 0.5 hours in growth medium. As seen in fig. 9A this generated a large population of replicating cells, about 50% of the total cell population was between 4N and 8N at the time of fixation. The second approach was to remove the growth medium and unattached trophozoites and replenish the stationary phase cells arrested in G2 with fresh growth medium, followed by growth for 2 hours. This also generated a large population of replicating cells as seen in fig. 9B, about 30% of the total cell population were between 4N and 8N at the time of fixation.

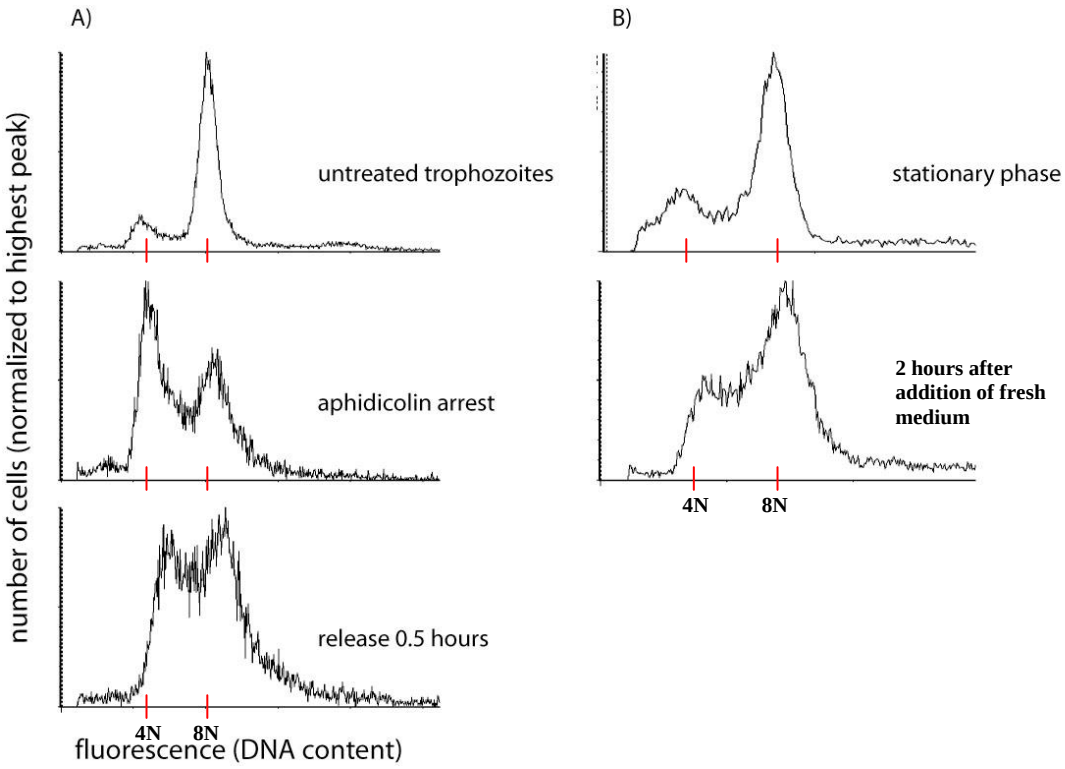

Fig. 9; Flow cytometry result for samples run on microarrays for marker frequency analysis. A) Normal population of trophozoites grown in TYDK medium. The second (aphidicolin arrest), cells grown in TYDKmedium and treated with aphidicolin. The third (release 0.5 hours) is aphidicolin treated cells and released after 30 minutes in new medium where about 50% of the total population were between 4N and 8N i.e. replicating population. B) First histogram shows cells grown to stationary phase in TYDK medium. The second, cells that grown in TYDK medium to stationary phase and re inoculated in fresh TYDK medium and grown for 2 hours, where about 30% of the total cell population were replicating and between 4N and 8N.

The DNA content in the replicating cells were compared to the DNA content in water treated cysts (16N). To analyse the samples, the arrays were scanned and the marker frequencies, calculated as the ratio between the two samples, were plotted for the different contigs of the published *Giardia* genome (contig 1, the longest assembled sequence, is presented in figure 10). This conting is approximately 870 kbp long, and should have several origins. If specific initiation sites were present one would expect a regular zig zag pattern in the plotted diagram representing the different origins. A ratio of 1 indicates that all cells have replicated that particular marker, whereas a ratio of 2 indicates that the cyst (with fully replicated genomes) have twice as much DNA corresponding to that marker; i.e. the cells in the replicating population have not yet replicated it. However, the marker frequency analysis did not identify any specific origins of replication in replicating *Giardia* trophozoites. There was no difference if drug had been used or not.

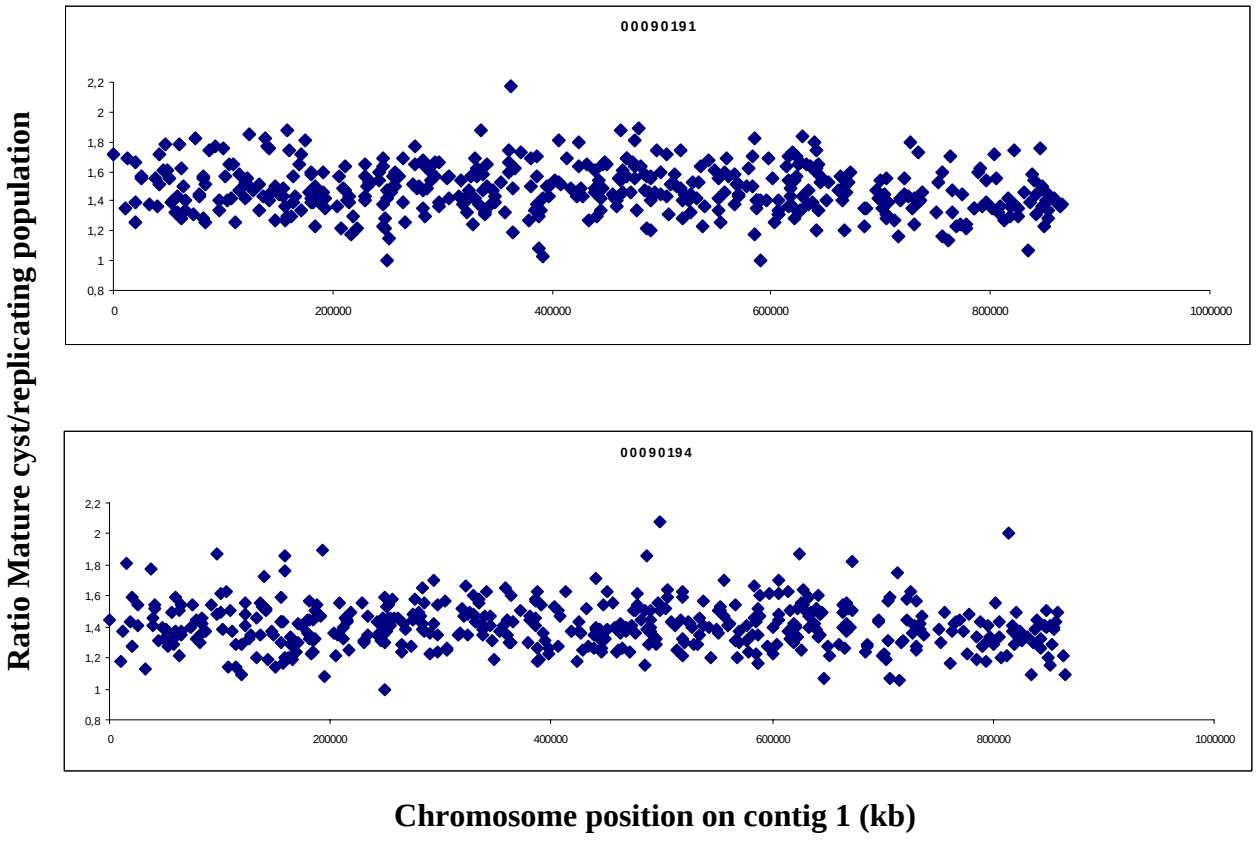

Fig. 10; Scatterplots of marker frequencies. Contig 1. 00090191 is the array with apidicolin treated trophozoites released for 0.5 hours in the S-phase hybridized against water treated cysts. 00090194 is the array with trophozoites that were grown to stationary phase and re-inoculated in new medium for 2 hours and hybridized against water treated cysts.

2.7 Fluorescence microscopy analysis of nocodazole treated cells

To see what morphological effect nocodazole had on the cells, they were stained with ethidium bromide and studied by microscopy. Many of the cells had altered shape and looked very irregular, often with a bump in the anterior part of the cell, see fig.11. Results from the flow cytometry with drugs had indicated that the cells treated with nocodazole at initiation of encystation had a 32N ploidy. Fluorescence microscopy was used to study these cell populations to see what morphological changes had appeared. Some of the trophozoites hade

more than 2 nuclei, up to 4 nuclei per cell was seen (fig. 11). Although the cells had been treated for 24 hours with encystation medium very few cysts could be seen (see fig. 12) and many cells were broken.

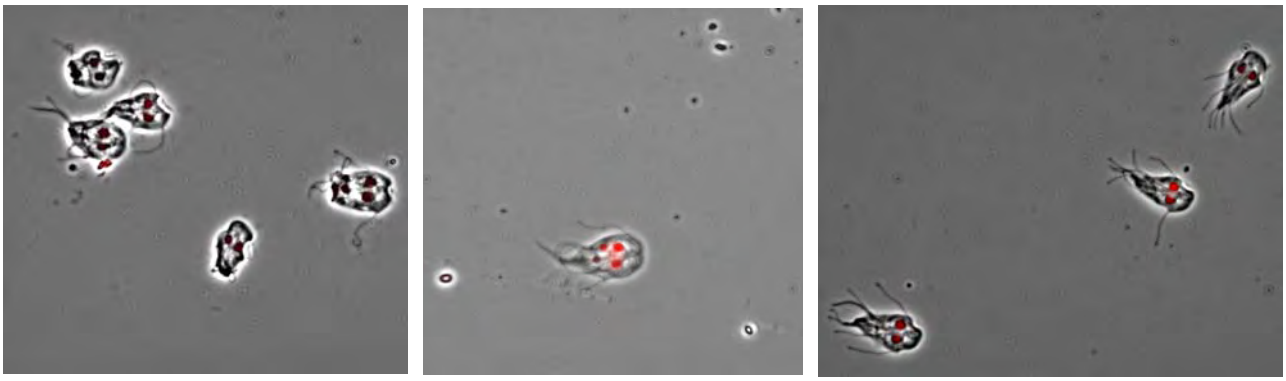

Fig. 11; Fluorescence microscopy picture of nocodazole treated cells. Cells were grown in TYDK- medium and treated with nocodazole during growth. The cells were then stained with ethidium bromide for fluorescence microscopy.

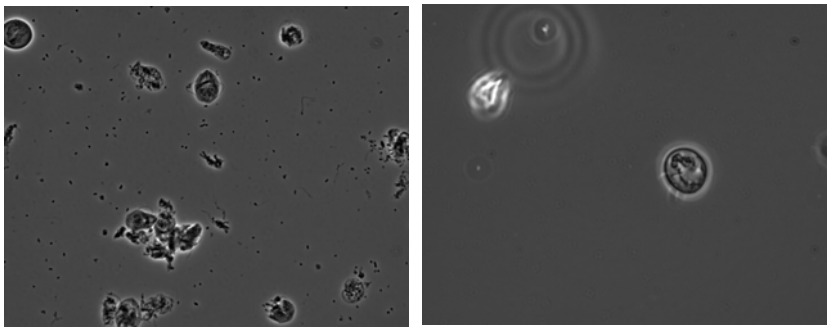

Fig. 12;. Fluorescence microscopy of nocodazole treated cysts. Cells were grown in TYDK-medium to full confluence and then encysted together with nocodazole for 24 hours. The cyst were stained with ethidium bromide.

3. Discussion

3.1 Encysting Giardia cells

The main aim of the study presented in this report was to survey the DNA replication during differentiation in *Giardia*. Earlier studies have shown that *Giardia* contains two approximately identically and synchronously replicating nuclei in the trophozoite (Wiesehahn *et al.* 1984; Tumova *et al.* 2007). Each of these nuclei cycle between a diploid 2N and a tetraploid 4N genome, giving a cellular ploidy of 4N and 8N (Svard *et al.* 2003). The replication of the DNA also has a highly important role during the encystation process in which the genome is replicated twice without cell division giving rise to a mature cyst with a ploidy of 16 N (Bernander *et al.* 2001). However, the nuclei are divided during the differentiation resulting in a single cyst with four tetraploid nuclei (Bernander *et al.* 2001).

My results showed that the replication from 8N to 16N starts around 12-15 hours after the initiation of encystation. After approximately 20 hours of encystation I could see that almost all cells had entered the replication phase. This shows that the DNA replication occurs in the late stage of encystation, when the cyst wall is already present. My results also indicate that the *Giardia* differentiation process (encystation) starts from the G2 stage of the cell cycle, which is also the case for other encysting eukaryotes (Bernander *et al.* 2001). However, multicellular eukaryotes usually differentiate from the G1 stage of the cell cycle (Svard *et al.* 2003).

The whole encystation procedure takes about 24 hours, depending on the conditions of the cells before the process starts. In the different experiments presented herein I sometimes observed less efficient encystation. One reason for this inefficient encystation could be contamination problems in the cell cultures. Another possible cause could be that the cell suspension had grown too thick, that is, there were too many cells in the tubes, before the initiation of the encystation. When *Giardia* grows to full confluence they reach a stationary phase where most cells arrest at late G2 (Reiner *et al.* 2008). It is then possible that fewer cells complete mitosis, G1 and S phase to reach the restriction point for differentiation.

The most widely used encystation method today is the protocol that uses bile starvation followed by addition of TYE medium. However, the results presented here indicate that this method is not the most effective to generate mature cysts. The results show that there still were cells with a 4N ploid after 48 hours. It was also evident that the cysts produced not all reached 16N but instead remined at 12N. Mature 16N cysts are necessary for good excystation *in vitro*, and completing the full *Giardia* life cycle. In comparison to the two other protocols presented here this is also a much slower encystation protocol. It involves two different encystation medium and takes at least 72 hours to complete after initiation of encystation. The procedure of changing medium one extra time also increases the risk of contamination as no antibiotics are used at any time. However, the comparison at 48 hours post initiation of encystation showed that the two other tested protocols are not suitable for such long encystation. What happens with the cells when they have encysted for such long time could be that the cells grew too many in the tubes and that the conditions for the cells in the tubes were not that optimal. When the cells are too many they are not able to attach the tube surface and the nutrients are not enough for all of them, making the environment far from optimal. The long exposure to the encystation media also could have a lethal effect on the cysts. The experiment with high bile went wrong somehow; the fixation for the flow analysis did not work as it should and very few cells could be analysed, maybe because of the high bile concentration.

3.2 Drug treatment during encystation

A second aim of the present work was to investigate the behavior of *Giardia* under the stress of the well-known cell cycle affecting drugs such as aphidicolin and nocodazole. Several studies have shown that different drugs can be used to affect the cells (Hoyne *et al.* 1989; Harris *et al.* 2001; Elmendorf *et al.* 2002; Mariante *et al.* 2005) but not until recently a method has been published describing whole culture synchronisation (Reiner *et al.* 2008). The choice of drugs for the different experiments presented here was based on their use as anti-giardial agents either in human or in veterinary medicine (Vesy & Peterson 1999; Harris *et al.* 2001) or on knowledge of direct or indirect effects on replication or mitosis (Dawson *et al.* 2007; Reiner *et al.* 2008).

The progression through the cell cycle depends on quality-control mechanisms called checkpoints. The checkpoints are points in the cell cycle where the cell monitors the correct completion of essential tasks, for example the completion of DNA synthesis, before it progresses to the next event in the cell cycle. One check point called the intra S-phase ensures that mitosis occurs only after DNA replication has been completed (Frouin *et al.* 2003).

It has been suggested that nocodazole has little or no effect against *Giardia* microtubules since the use of this drug did not affect attachment of parasites (Elmendorf *et al.* 2002). However, those results are only based on the indirect attachment effect, which means that nocodazole effected the *Giardia* trophozoite in that it couldn't attach to the surface. In contrast to the results in the attachment study, nocodazole was seen to have a dramatic effect on cells in other studies (Mariante *et al.* 2005; Dawson *et al.* 2007). These results showed that cells treated with nocodazole became totally misshapen, with morphological changes such as abnormal numbers of flagella, irregular dorsal surface, membrane blebs, distruption of the lateral flange, and loss of normal morphology (Mariante *et al.* 2005). Other changes observed were the effect on the microtubule dynamics in dividing cells resulting in broken spindles and various chromosome segregation defects (Sagolla *et al.* 2006). Tubulin is a dominant protein in *Giardia*. There are two alphatubulin and three betatubulin genes. In all known eukaryotic cells microtubuli are formed from alpha- and beta tubulins (Mariante *et al.* 2005).

In agreement with those latter studies my results indicate that nocodazole seriously affects the *Giardia* cells. Nocodazole seems to have an effect on the cells in that they do not divide but they continue their DNA replication, which results in cells with up to 32N. The results (see fig. 7) also indicate that when nocodazole was added at 3 hours post initiation of encystation a majority of the cells reached 16N, which was not the case either in the encystation control or when nocodazole was added later. Most likely, these cells are not mature cysts but trophozoites that have been blocked in cell division but manage to pass through the spindle assembly checkpoint, resulting in endoreduplication. This effect of the drug would not be seen at 6 hours or later as all cells then would have had time to complete a full cell cycle (Reiner *et al.* 2008) and pass the restriction point for encystation and start to encyst as destined. The effect of single G1 cells containing more than a normal genomic setup has been described earlier in both *Giardia* (Davids *et al.* 2007) as well as in other organisms (Rieder & Maiato 2004).

In earlier studies *in vitro* of macromolecular synthesis it has been seen that only DNA synthesis is sensitive to aphidicolin. A study on seaurchin embryos showed that only DNA polymerase alpha is sensitive to aphidicolin (Oguro *et al.* 1979). This study also showed that the inhibiton of DNA polymerase alpha by aphidicolin was due to its competition with dCTP.

One possible explanation is that aphidicolin binds to the dCTP site and thereby inhibits DNA polymerase alpha activity. Alternatively aphidicolin binds to a site other than the catalytic sites and competes with dCTP by a yet unknown mechanism (Oguro *et al.* 1979). Recently, aphidicolin was described as a reversible inhibitor of giardial DNA replication (Reiner *et al.* 2008). In the study presented here I used this drug during encystation to determine its effect on encystation and the endoreduplication that takes place during the differentiation. My results clearly showed that cell cultures treated with aphidicolin at the initiation of encystation and at some point during the first 12 hours of encystation were affected by the drug and did not produce 16N cysts. The expected results would be that almost all cells would be stuck at 4N. However, as seen in figures 6, 7 and 9 many or most cells reached 8N. This could be either cells that were in the S-phase when aphidicolin was added or cells that managed to escape the drug after some time. If the cells had entered S-phase but not completed the final stage of the DNA replication, the cells could have been arrested at the G2/M check point (Reiner *et al.* 2008). After 15 hours treatment aphidicolin seemed to have lost its effect to block the DNA replication as cyst hade been formed.

Cells treated with flagyl did not seem to be affected in their DNA content at all (see fig. 6). This could indicate that flagyl is not as effective in inhibiting the differentiation and development of *Giardia* as many other drugs (Hausen, Jr. *et al.* 2006). Another reason for the lack of effect could be that flagyl does not affect differentiation, but only affect the vegetative cells. This is in agreement with earlier studies where no effect on cysts could be observed (Harris *et al.* 2001). The absence of effects on the DNA could also be explained by the drug's action, which might not be reflected by the DNA content. Since these samples were stained only for DNA, all other effects might have been missed. Flagyl is often used in medicine together with some other antibiotic, for example albendazole (Upcroft & Upcroft 2001). Albendazole, which is a tubulin inhibitor, has similar effect on the cells as nocodazole see fig. 6. This means that the cells treated with albendazole continue their DNA replication even when the cell division is stopped, as seen in fig 6.this gives a result with cells that have more than two nuclei.

3.3 Marker frequency

DNA replication in eukaryotic cells is a tightly regulated process and leads to the duplication of the genetic information for the next cell generation. The events involved in chromosomal replication starts with the binding of specific initiator proteins to DNA sites, termed origins, and results in the establishment of replication forks. *Giardia* has fewer replication proteins than for example yeast (Morrison *et al.* 2007). In bacteria and archea the replication starts from one or more specific sites on the genome that serves as an origin in every cell cycle (Lundgren *et al.* 2004; Zakrzewska-Czerwinska *et al.* 2007). On the other hand, most eukaryotes have multiple chromosomes, each with multiple replication origins (Prasanth *et al.* 2004; Robinson & Bell 2007). In the present study no origin of replication could be identified. This is in agreement with results from other studies on origin of replication in eukaryotes. One explanation that has been suggested is that eukaryotic cells are replicated from several origins with flexibility in origin selection and firing (Jeon *et al.* 2005). In *Giardia* plasmids can replicate without a specific origin of replication and our results with non-specific origins of replication are in agreement with this. As DNA replication is essential for both the trophozoite and the formation of mature cysts the mechanisms involved may provide valuable drug targets against *Giardia* infection and transmission.

4. Materials and methods

4.1 Strains and cell maintenance

Giardia lamblia trophozoites, strains WBC6 were cultivated in 10 ml Nunclon delta flat side tubes (NUNC, prod. no. 156758) in TYDK medium at 37˚C. The cells were re-inoculated every four days. To re-inoculate the cells, the cells were detached from the tube wall by chilling the tubes for 10-15 minutes on ice.

TYDK medium: For 500 ml: 15 g peptone, 5 g glucose, 1 g NaCl, 0.50 g KH_2PO_4 $3H_2O$, 0.30 g KH_2PO_4, 1 g L-cysteine, 0.10 g L-ascorbic acid, 25 mg ferric ammonium citrate were mixed with sterile water up to 445 ml in a glas pot, while pH were measured with PHM210 standard pH meter and adjusted to 7.1 by adding some drops of 4M NaOH. The medium were filtered through a 0.45µm filter (Sigma). Then 50 ml sterile bovine serum was added. To make 25 X filtered bovine bile, 0.65 g bovine bile (Sigma) was dissolved in 50 ml sterile water and filtered thru a 0.45µm filter. 5 ml of this was added to the medium.

4.2 Cyst culture

4.2.1 Cyst culture with encysting medium and encysting medium with high bile

The medium was poured off cells (75-100% confluence) and replaced by the encysting medium.

Encysting medium: For 500 ml: 15 g peptone, 5 g glucose, 1 g NaCl, 0.50 g $KH_2PO_4 \cdot 3H_2O$, 0.30 g KH_2PO_4, 1 g L-cysteine, 0.10 g L-ascorbic acid, 25 mg ferric ammonium citrate were mixed with sterile water up to 425 ml in a glas pot, while pH were measured with PHM210 standard pH meter and adjusted to 7.8 by adding some drops of 4M NaOH. The medium was filtered through a 0.45µm filter (Sigma). Then 50 ml sterile bovine serum was added. To make bovine bile, 2.5 g bovine bile was dissolved in 50 ml sterile water and filtered thru a 0.45µm filter. 25 ml of this was added to the medium.

Encysting medium with high bile: For 500 ml: the same recipe as for the encysting medium but the volume of the medium mix before adding serum and bile were 400 ml, because double amount of bile were added. 50 ml filtered bile (2.5 g bovine bile in 50 ml sterile water).

4.2.2 Encysting cells with TYE-medium

Starvation medium without bile, was the same recipe as for TYDK-medium but without bile.

TYE-Medium: For 250 ml TYE-medium: 7.5 g peptone, 2.5 g glucose, 0.5 g NaCl, 0.15 g KH_2PO_4, K_2HPO_4, 0.5 g L-cysteine, 0.05 g L-asorbic acid, sterile water up to the volume of 206 ml were mixed together in a glas pot. pH was messured with PHM210 standard ph meter, Meter lab and measured to 7.8 by adding drops of 4M NaOH. The medium mix was filtered thru a 0.45µm filter. Then 25 ml sterile bovine serum were added and 6.25 ml porcine bile (0.125 g porcine bile (Sigma) in 25 ml sterile water filtered thru 0.45µm filter (Sigma)) and 12.5 ml lactic acid (0.275 g lactic acid in 25 ml sterile water filtered thru 0.45µm filter (Sigma)).

4.2.3 Water treated cysts

The tube where the cells hade encysted was centrifuged at 1500 g, then the encysting medium was removed and 10 ml sterile water was added, the pellet was resuspended and the mix incubated at 4°C for 24h. The sample was centrifuged for 5 min at 1500 g, the supernatant was removed and the pellet was washed again with 5 ml sterile water. After a final centrifugation the supernatant was removed and the pellet was further used for flow cytometry or DNA extraction.

4.2.4 Cells treated with drugs during encystations
Cells treated with drugs during encystations were first grown to full confluence in TYDK medium and when the medium was changed to encysting medium the drug was added [5µl (10 mg/ml) apidicolin, 3µl (10 mg/ml) nocodazole (Fluka), or 10µl (10 mg/ml) Albendazole and 10µl (10 mg/ml) Flagyl (Sigma)].

4.3 Fixation before flow cytometry analysis
The samples form the encysted cells were collected either by scraping the tubes or by incubation on ice for 10-15 minutes. The tubes were then centrifuged for 5 minutes, 1500 g at 4ºC. The supernatants were removed. The pellet was fixed with 150 µl fixation solution (10 ml of 10% Triton X 100, 39.8 ml of 0.1 M citric acid, 10.2 ml of 0.2 M dibasic sodium phosphate, 20 ml of 1 M sucrose, and 20 ml of distilled water, pH 3.0 measured with a pH-meter and 4M NaOH), incubated at room temperature for 5 min, and then 350 µl diluent buffer (125 mM $MgCl_2$ in phosphate buffer saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 pH 7.4) was added to each tube (Dvorak 1993).

4:3:1 Wash before flow cytometer analysis
The cell pellets that had been fixed were transferred to eppendorf tubes and centrifuged 3 minutes at 3500 g. The supernatant was removed and 100 µl PBS added. Samples were mixed gently, and centrifuged again 3 minutes at 3500 g. The supernatant was removed and 500 µl PBS and 2.5 µl RNase A (10 mg/ml) were added to each sample, which was gently mixed. The samples were incubated for 30 minutes at 37 ºC.

4.3.2 Labeling of DNA before flow cytometry
The sample was centrifuged 3 minutes at 3500 g. The supernatant was removed and 75 µl of the labeling color mix of 0.75 µl (4 mg/ml) ethidium bromide (EtBr), 6 µl (2.5 mg/ml) mitramycin A and 68.25 µl of Tris-$MgCl_2$ (10 mM TrisHCl pH 7.4, 10 mM $MgCl_2$) was added to each sample, which was gently mixed. 75 µl of Tris-$MgCl_2$ was then added to each sample, which was mixed again. The sample was incubated on ice for 20-30 minutes before being analyzed by flow cytometry.

4:4 *Giardia lamblia* micro arrays
Whole genome microarrays were supplied by TIGR, Pathogen Functional Genomics Resource Center, PFGRC. The arrays are oligo-arrayswith 9115 70-mer oligo printed in duplicates onn each array.

4:4:1 Extraction of genomic DNA from cultured Giardia
Cells were transferred to a 1.5-ml centrifuge tube and were collected in a pellet by centrifugation 3 min at 3500 g. The pellet was then resuspended completely by pipetting in 500µl of buffer (50 mM EDTA, 1 % SDS). In the extraction of DNA from cysts an extra step was included to break the tough cyst walls by disrupting the cyst with 0.5 mm Silica beads (BioSpec Products Inc.) 2 x 30 seconds in 500 µl buffer (50 mM EDTA, 1 % sodium dodecyl sulphate (SDS)). Trophozoites were lysed directly in 500 µl buffer (50 mM EDTA, 1 % SDS). A volume of 14 µl Proteinase K (20 mg/ml) was added to the cell suspension, which was mixed. The tubes were then incubated for 1 hour at 56°C and occasionally vortexed. To get an RNA-free sample, 4 µl RNase A (100 mg/ml) was added, and the sample was then incubated at room temperature for 5 minutes. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the DNA solution and mixed. The samples were then centrifuged for 10 minutes at 3500 g. After centrifugation the upper parts were transferred to new tubes and an equal volume of Chisam (chloroform:isoamyl alcohol 24:1) was added and

mixed. The tubes were then centrifuged again for 10 minutes at 3500 g. The upper parts were transferred to a new tube and an equal volume of isopropanol was added, the samples were incubated at room temperature for 10 min, and then centrifuged for 10 minutes at 3500 g. After centrifugation, the supernatants were removed and 1 ml of 70% cold ethanol was added to wash the pellet. The ethanol was removed and the samples were left to dry at room temperature. 21 µl water was then used to dissolve the pellet by pipetting and the OD of the solution was measured at 260 nm.

4.4.2 DNA Labeling

Purified DNA (2 µg) was mixed with 100 pmol of random nonamer oligonucleotides (Operon) in a final volume of 10 µl. The DNA was denatured for 5 min at 95°C, followed by incubation on ice for 2 min. Labeling mix (10 µl) containing 2× REact 2; 0.5 mM (Invitrogen) each of dATP, dCTP, and dGTP (Invitrogen); 0.2 mM dTTP (Invitrogen); 4 mM potassium phosphate buffer (pH 8.0); 0.1 mM Cy3/Cy5 dUTP (Invitrogen); and 5 units of DNA polymerase I Klenow fragment (Invitrogen) was added, and the samples were incubated for 2 h at 37°C. Samples to be cohybridized were mixed and purified by using a Minelute kit (Qiagen) and eluted in 2 × 10 µl of the elution buffer provided with the kit.

4.4.3 Prehybridisation

The micro array was placed in 42^{o}C prehybridisation solution (0.5 g bovine serum albumin (BSA) in 50 ml of 5x sodium-saline citrate (SSC) (0.75 M NaCl and 0.075 M sodium citrate), 0.1% SDS and incubated for 40 minutes at 42^{o}C. Then the slides were dipped in three different Coplin jars with ddH_2O followed by once in one with isopropanol. The slides were then dried gently by centrifuging at 300 g in a table top centrifuge.

4.4.4 Hybridisation

The labeled DNA was mixed with 60 µl of hybridization mixture (63% formamide, 6× SSC, 0.16% SDS, 10 µg of tRNA (Sigma), and 10 µg of herring sperm DNA (Sigma)). The samples were denatured at 95°C for 2 min and then incubated on ice. The array was placed in a hybridization cassette (humidified with 20 µl of H_2O) and a coverslip was applied. The hybridization mixture was added. The reaction was incubated in the dark for 20h at 42°C. After hybridization, the arrays were washed for 5 min in 2× SSC and 0.1% SDS at 42°C, then for 10 min in 0.1× SSC and 0.1% SDS at room temperature, and finally 5 times in 0.1× SSC at room temperature. The slides were then dried gently by centrifuging at 300 g in a table top centifuge. The arrays were scanned at 10-µm resolution by using an Agilent micro array scanner model G2565 BA (Aglient Technologies, Palo Alto, CA) at 635 and 532 nm.

4:5 Microscopy

To see how the cells looked after treatment with nocodazole they were fixated with para-formaldehyde (PFA) fixation and stained with ethidium bromide.

4:5:1 Fixation of cells

Cells were grown in 10 ml flat-side tube (NUNC) to full confluence and treated with 3 µl (10 mg/ml) of nocodazole for 24 hours. The cells were detached from the tube by incubation on ice for 30 min. Then the cells were transferred into 15 ml falcon tubes, and centrifuged 5 minutes at 4ºC, 1500 g. To make the cells more active and able to reattach to a surface, they were resuspended in TYDK pre-warmed to 37ºC at a concentration of 10^6 cells/ml. The different cell suspensions (10 µl) were placed in different 6 mm-well on diagnostic microscope slides. The slides were then placed in a humidity chamber (a small plastic box humidified with wet paper) for 20 min, to allow the trophozoites to attach to the slide surface.

The slides were then washed twice with warm PBS, and put back in to the humidity chamber to dry slightly. For fixation 10 µl of 2 % paraformaldehyde (PFA) was used in each well. During fixation the slides were incubated at 37ºC for 20 minutes. The slides were then washed with PBS and 10 µl 0.2 % PFA was added to dry at room temperature.

4.5.2 Staining of cells

The wells covered with 0.2 % PFA were rinsed once with PBS. Subsequently they were incubated with PBS and 0.1 % Triton X-100 at room temperature for 30 minutes to generate permeabilized membrane. The slides were washed once more in PBS and left to dry slightly at room temperature. The cells were stained with 10 µl 0.01 %EtBr to a volume of 10^6 cells/ml. To remove the background staining, the wells were washed three times with PB. 5 µl of 50 % glycerol was added in each well. To keep the coverslip in firm position nail polish was used as glue.

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