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Summary of Ph. D. Thesis

CELL CYCLE AND DIFFERENTIATION IN *GIARDIA INTESTINALIS*

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CONTENT:

ABSTRAKT

Giardia je jednobuněčný parazitický organismus, který je zdrojem průjmových onemocnění po celém světě. Má minimální genomovou výbavu a zjednodušené molekulární a metabolické dráhy. V tomto ohledu je to vhodný modelový organismus pro studium regulace buněčného cyklu a pro definici minimální genetické a proteinové výbavy nutné pro reprodukci eukaryotické buňky. V životním cyklu giardie se vyskytují dvě stádia: patogenní trofozoit a infekční cysta, která dokáže přežít ve vnějších podmínkách. Nové znalosti o encystaci mohou být významné z terapeutického hlediska, protože na tento proces je zacílen vývoj vakcíny a léků. Protože studium buněčného cyklu vyžaduje synchronizovanou populaci, studovali jsme vliv synchronizační látky aphidicolinu na jednotlivé buněčné děje během buněčného cyklu trofozoitů giardie. Naše výsledky ukázaly, že aphidicolin zastavil syntézu DNA a trofozoiti byli zablokováni podle obsahu DNA na hranici G1/S fáze. Následná inhibice vstupu do mitózy a cytokineze naznačuje, že *Giardia* má funkční kontrolní bod při poškozené DNA. Aphidicolin působí při dlouhodobé inkubaci a vyšších koncentracích vedlejší efekty; detekovali jsme pozitivní signál pro fosforylovaný histon H2A, který je u savčích buněk součástí signalizační dráhy spuštěné jako reakce na dvouřetězcové zlomy v DNA. Reverzibilita této posttranslační modifikace po odstranění inhibitoru ukazuje, že *Giardia* má reparační mechanismy poškození DNA. Inkubace s aphidicolinem působí disociaci jaderného a cytoplazmatického cyklu. Zatímco syntéza DNA a vstup do mitózy jsou zastaveny, cytoplazmatický cyklus pokračuje. Zaměřili jsme se také na popis jaderného dělení během encystace ve vztahu k tvorbě cystové stěny a přestavbě bičíkového aparátu. Jaderné dělení proběhlo semi-open mitózou za účasti dvou mitotických vřetének v rané fázi encystace před vznikem cystové stěny. Jádra zůstala po rozdělení v párech a byla propojená několika můstky, které vznikly fůzí jaderných membrán. Každý propojený pár jader je spojený s jednou tetrádou bazálních tělísek nerozděleného mastigontu diplomonády.

ABSTRACT

Giardia is a unicellular parasitic organism; it is a worldwide cause of human diarrhea. It has minimalistic genome equipment and simplified molecular and metabolic pathways. In this respect, it is a suitable model organism for studying cell cycle regulation and to define the minimal genetic and protein equipment required for the functional reproduction of the eukaryotic cell. Its life cycle comprises of two stages; a pathogenic trophozoite and an infective cyst, which can survive in outer environment. New knowledge about encystation can be therapeutically important because this process is a target for vaccine and drug development. Since cell cycle analysis requires a synchronized population, we studied the effect of the synchronization drug aphidicolin on individual cell characteristics during the cell cycle of *Giardia* trophozoites. Our results showed that aphidicolin caused inhibition of DNA synthesis and trophozoites were aligned according to their DNA content in G1/S border. Subsequent inhibition of entry into mitosis and cytokinesis indicates, that *Giardia* has functioning DNA damage checkpoint. Extensive treatment with aphidicolin causes side effects. We detected positive signals for phosphorylated histone H2A which, in mammalian cells, is involved in a signaling pathway triggered as a reaction to double stranded DNA breaks. Reversibility of this posttranslational modification after inhibitor removal indicates that *Giardia* possesses DNA damage reparatory mechanisms. Aphidicolin treatment causes dissociation of the nuclear and cytoplasmic cycles. While DNA synthesis and entry to mitosis are stopped, the cytoplasmic cycle and its processes continue. We also focused on characterization of nuclear division during *Giardia* encystation with respect to cyst wall formation and flagellar apparatus arrangement. Nuclei are divided by semi-open mitosis in early phase of encystation in a precyst before cyst wall formation. After the karyokinesis, nuclei stayed in pairs and were interconnected with several inter-nuclear bridges formed by fusion of nuclear envelopes. Each interconnected nuclear pair is associated with one basal body tetrad of the undivided diplomonad mastigont.

1. INTRODUCTION

Giardia is a widespread cause of diarrhea and it was included in the "Neglected Disease Initiative" of the WHO (Savioli et al. 2006). In the last 20 years *Giardia* is also attractive from the evolutionary point of view, as it was considered to be among one of the earliest branching eukaryotes. Together with other Metamonada and putatively amitochondriate groups like Parabasala, Microsporidia and Archamoeba, it was placed to Archezoa (Cavalier-Smith 1989).

With ongoing genome project in *Giardia,* putative mitochondria proteins were discovered as well as double membrane organelle called mitosome (Tovar et al. 2003). *Giardia* however retains some primitive characteristics: absence of peroxisomes, typical Golgi apparatus and myosin, it encodes for the bacterial arginine pathway and contains only few introns in its compact genome (Morrison et al. 2007).

The trophozoite is a pear-shaped octo-flagellated cell approximately 12-15 μm long and 5-9 μm wide. The plasma membrane of *Giardia* trophozoite is covered with a coat from variable-specific surface proteins (VSP). *Giardia* like other diplomonads possesses two morphologically indistinguishable nuclei. Nuclei are positioned with mirror symmetry with respect to long axis in the anterior part of the cell adjacent to two basal bodies tetrads. Ventral surface of trophozoite is modified into an adhesive disc. To the posterior end from adhesive disc is situated a rod-shaped median body. *Giardia* has a complex of basal body - associated structures constituting a mastigont, which comprises 8 flagella, supranuclear microtubules extending to an adhesive disc, funis, dense rods and striated lamellae. Eight flagella, with canonical eukaryotic microtubular structure 9+2, are organized in four pairs: anteriolateral, posteriolateral, caudal and ventral (Kulda and Nohýnková 1995).

Up to recently, cell cycle regulation in parasitic protists was not well understood. Cell cycle regulation in these organisms has many different features from canonical regulation in eukaryotic cells, because of their complex life cycles and ability to live in changing environmental conditions (Hammarton et al. 2003). The completed *Giardia* genome project of assemblage A (isolate WB) http://giardiadb.org/giardiadb/, to which assemblages B (isolate GS) and assemblage E (isolate P15) were recently added, provides information about cell cycle regulatory gene equipment in *Giardia*. *Giardia* has one of the smallest genomes in eukaryotes - 12 Mb for haploid genome. Though many cell cycle regulatory pathways are simplified, the cell cycle in *Giardia* is regulated with the conserved regulatory mechanisms as in typical eukaryotic cell (Morrison et al. 2007). *Giardia* proceeds through all four typical

eukaryotic cell cycle phases. The course of mitosis in *Giardia* was described recently. Chromosomes condense in early prophase. Each nucleus is divided by a single mitotic spindle. The nuclear envelope does not break apart during mitosis in *Giardia*, only some of the extranuclear mitotic spindle microtubules enter the nuclear envelope. The type of *Giardia* mitosis has been described as semi-open orthomitosis according to Raikov (1994); (Sagolla et al. 2006; Tůmová et al. 2007b).

The nuclei are distributed into daughter cells equationally: each daughter cell receives one copy of each mother nucleus (Yu et al. 2002). Eight parent flagella are equally segregated between two daughter cells and four new flagella are formed *de novo*, however each daughter cell receives different half set of mother flagella (Nohýnková et al. 2006).

Giardia undergoes differentiation, which comprises encystation and excystation. The four nucleated cyst has protective cyst wall formed from carbohydrates (β-(1-3) linked N-acetyl galactosamine) (Karr and Jarroll 2004) and proteins (cyst wall proteins - CWP1 – 3 and high cysteine non-variant cyst protein (HCNCp). Proteins forming the cyst wall are upregulated only during encystation and they are transported to nascent cyst wall in encystation specific vesicles (ESV), which are considered to be Golgi-like compartments (Lujan et al. 1995; Sun et al. 2003; Davids et al. 2006; Stefanic et al. 2009). Nuclei division during encystation is elusive and basic description is missing. Recently, Poxleitner et al. (2008b) showed signs of genetic recombination as a result of nuclear fusion between two non-daughter nuclei in cyst. The question of possible sexual recombination in *Giardia* has been raised because of low level of allelic sequence heterozygosity (ASH) <0.01% in genotype A in *Giardia* WB isolate (Adam 2001). Finding of 17 homologues coding the “core meiotic recombination machinery” that function in meiosis in higher eukaryotes in *Giardia* genome, supported the idea that meiosis may still be operating in *Giardia*, otherwise these proteins would not be retained in genome (Ramesh et al. 2005).

2. HYPOTHESIS AND AIMS OF THE THESIS

Giardia intestinalis was considered one of the earliest branching eukaryotic organisms. Though the discovery of mitochondrial genes in its genome and description of mitosomes showed it is not as primitive as originally believed, it still possesses some primitive characteristics. It has an unusual cell structure with two nearly identical nuclei and complex cytoskeleton, a small genome and minimalistic genome equipment, and simplified molecular and metabolic pathways. In this respect, it is a promising model organism for studying cell

cycle regulation and to define the minimal genetic and protein equipment required for the functional reproduction of the eukaryotic cell. Studying cell cycle regulatory molecules requires a synchronized population, which was not available in *Giardia*. We studied more thoroughly the influence of one DNA replication inhibitor, mycotoxin aphidicolin, recently used as a tool for *Giardia* synchronization. *Giardia* also represents a health risk, being a worldwide cause of diarrhea in humans, and it is important to understand the mechanisms of encystation as the prerequisite for transition into a new host. Despite of great expansion in biochemical and molecular methods, which enabled the characterization of single molecules during differentiation and has provided great insight about cyst wall formation, the basic description of nuclei behavior and the flagellar apparatus reorganization during encystation and excystation is still unknown. Possible exchange of genetic material in cyst and the presence of meiotic genes in *Giardia* genome along with prediction of sexual process in *Giardia* focus even more the attention on the enigmatic nuclei division during encystation.

OBJECTIVES:

- To study the effect and reversibility of the synchronization drug aphidicolin on individual cell characteristics during the cell cycle of trophozoites. The purpose of this study was to determine the extent and for which parameters the population can be regarded as synchronized.
- To characterize individual phases of encystation in respect to cyst wall formation in *Giardia,* to identify the stage of encystation when the nuclei divides and determine how these two processes are interconnected.
- To find out which type of mitosis occurs in encystation, how the mitotic spindle is nucleated. And to further follow the behavior of flagellar apparatus, which is tightly coordinated with karyokinesis in *Giardia.*

3. MATERIAL AND METHODS

The materials and methods were described in details in the published articles.

Cell cultures, encystation, excystation

The HP-1 line of the Portland-1 isolate of *Giardia intestinalis* (ATCC 30888), donated by E. A. Meyer (Oregon Health Sciences University, Portland, USA) was used. Trophozoites were grown axenically in a filter-sterilized TYI-S-33 medium, pH 6.8, supplemented with bovine bile (Keister 1983) at 37°C. Encystation was induced by an encystation protocol

adapted by McCaffery and Gillin (1994). Encystation medium TYE-GS3, pH 7.8 was enriched with bile bovine (5 mg/ml) and calcium L-lactate (0.5 mg/ml). Excystation was induced in cysts isolated from either human or sheep stool protocol adapted by Hetsko et al. (1998). Cysts were resuspended in the low pH suspension (pH 4.0) containing reduced glutathione and L-Cysteine in Hank´s balanced solution then in solution (pH 8.0), containing Trypsin and taorocholic acid in Tyrode´s salt solution.

Immunofluorescence labeling

Single labeling in trophozoites and excyzoites

Immunofluorescence staining was carried out according to Nohýnková et al. (2000). Trophozoites were allowed to adhere on cover slips and fixed with ice-cold methanol, permeabilised with ice-cold acetone, rehydrated in phosphate-buffer saline (PBS) at pH 7.2 and blocked with 3% bovine serum albumin BSA/PBS. They were incubated with primary antibody in 2% BSA/0.1% Triton-X-100/PBS, washed with PBS pH 7.2 and incubated with secondary antibody in 2% BSA/0.1% Triton-X-100/PBS, washed with PBS pH 7.2 and finally mounted in Vectashield mounting media with DAPI (Vector).

Double labeling

Cysts were harvested by centrifugation, washed in PBS, centrifuged and allowed to adhere on cover slips and air-dried. Cysts were further fixed with ice-cold methanol, permeabilised with ice-cold acetone, rehydrated in PBS pH 7.2 and blocked with 3% BSA/PBS. Samples were further incubated primary antibody in 2% BSA/0.1% Triton-X-100/PBS, washed with PBS pH 7.2 and incubated with secondary antibody in 2% BSA/0.1% Triton-X-100/PBS, washed with PBS pH 7.2. Slides were then post-fixed in 4% paraformaldehyde and washed with 100 mM glycine/PBS. The second round of blocation was performed with 2% BSA/PBS and then the second antibody against cyst wall protein 1 A300 Cy3 in 2% BSA/0.1% Triton-X-100/PBS was added, washed with PBS and finally mounted in Vectashield mounting media with DAPI.

Electron microscopy

Suspensions of encysting cells after 24 hrs incubation in TYE-GS3, cysts prepared *in vitro* and cysts of human or sheep origin were spinned and resuspended in cold fixative (2.5% glutaraldehyde/5 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.4) and fixed overnight. Fixed cells were washed with PBS, pH 7.2, post-fixed in OsO_4/0.8% potassium ferricyanide/5 mM $CaCl_2$ in the same buffer, thoroughly washed with cold PBS, pH 7.2, dehydrated in acetone,

and embedded in Epon. Ultrathin sections were examined after staining with uranyl acetate/lead citrate with a JOEL 1010 electron microscope.

Flow cytometry analysis

Cells were resuspended in fresh TYI-S-33 and fixative. Then diluent buffer was added and samples were stored at 4ºC until measurement. Fixed cells were washed in PBS and resuspended in PBS with 1µg RNAse A. Then, cells were resuspended in 10 mM Tris/10 mM $MgCl_2$ with 10 µg/ml propidium iodide and stained. Flow cytometry was performed on FACS Canto II (BD Biosciences). The data were analyzed using BD FACSDiva^{TM} software.

4. RESULTS

Aphidicolin influence on cell cycle

Studying cell cycle regulatory molecules requires a synchronized population. Synchronization of cell population may be achieved by different approaches. It is likely that this is most often achieved by inhibiting either DNA replication or nuclei division using specific inhibitors. We studied the influence of the DNA replication inhibitor, aphidicolin on *Giardia* trophozoites.

The influence of aphidicolin on the *Giardia* cell cycle is both dose and time dependent. The working concentration of 5 µg/ml was adjusted, based on the initial experiments. DNA content of propidium iodide (PI) stained trophozoites in logarithmic phase of growth was measured by flow cytometry. We found that approximately 70% of untreated cells resided in G2/M corresponding to DNA content 8N and the rest of population was in G1/S phase (corresponding to 4N). In aphidicolin treated cells, the DNA content of the majority of the cell population shifted to the G1/S junction. Incorporation of BrdU provided direct proof that DNA synthesis was disrupted by aphidicolin treatment. A positive signal for BrdU incorporation, representing the rate of DNA synthesis, was detected in either one or both nuclei in about 33% of untreated *Giardia* cells. The DNA was replicated asynchronously as demonstrated by asymmetry in fluorescence signal between the two nuclei. In 10% of the cells, only one of the two nuclei showed a positive signal for BrdU incorporation. Phosphorylated form of histone H2A, a modification of H2A, which is essential in DSB recognition and their repair (Zhou and Elledge 2000) was present in one or both nuclei in 55% of cells already after short incubation with aphidicolin. The presence of signal in only one nucleus might again correspond to asynchronicity in DNA synthesis between the nuclei. The presence of signal in both nuclei increased with prolonged incubation and reached 99% after

48 hours. Its presence was however reversible and decreased after release from aphidicolin block. The fluorescence signal for phosphorylated H2A occurred also naturally in untreated trophozoites, though only as single dots and usually in only one of the two nuclei in comparison with full signal in aphidicolin treated cells. Another side effect of aphidicolin treatment was the causative dissociation of nuclear and cytoplasmic cycles. While the nuclear cycle was stopped at the G1/S border, the cytoplasmic cycle and its processes (protein synthesis, increase in cell size) continued. *Giardia* trophozoites grew significantly in cell size with prolonged incubation with aphidicolin. Correspondingly, these trophozoites contained almost in 100% median bodies, which are regarded as G2 marker, and these were bigger than in untreated G2 cells.

Albendazole influence on cell cycle

A microtubular polymerization inhibitor albendazole prevented nuclei division in *Giardia* by blocking the mitotic spindle assembly. However, the cytokinesis continued in most cells resulting in aberrant trophozoites containing either none, one or two nuclei. After 24 hours of incubation with albendazole, three distinctive peaks were formed on cytometry histograms, one corresponded to G1/S, one approximately to G2 and one was situated in between 8N and 16N DNA content. The second round of DNA replication in nuclei that did not undergo karyokinesis was further documented with BrdU incorporation and chromosomal spreads, which showed even twenty condensed chromosomes per nucleus (average number per nucleus in typical mitotic cell was ten).

Nuclear division and DNA replication during encystation

The completion of *Giardia* life cycle is crucial for its successful transmission to a new host. The whole life cycle of *Giardia* can be performed *in vitro* and therefore, processes occurring during encystation and excystation can be studied. Our studies of encystation and excystation were focused on nuclei division, DNA replication and flagellar apparatus reorganization in both processes. To follow DNA replication during differentiation, we performed flow cytometry analysis of DNA content on PI stained cells, BrdU incorporation and the inhibitory effects of albendazole.

DNA content of *in vitro* gained cysts, measured by flow cytometry, confirmed that cysts had double the amount of DNA than trophozoites in G2 phase, corresponding to 16N as stated previously by Bernarder et al. (2001). To determine the timing of DNA replication during encystation, we performed BrdU incorporation in encysting cells. Positive staining for BrdU occurred in 36% of cysts. In all BrdU positive cysts, all four nuclei showed BrdU signal.

DNA replication therefore occurred in each nucleus and followed the nuclear division. Albendazole treatment prevented nuclei division, which indicated the presence of microtubular mitotic spindle in nuclear division during encystation. Albendazole however did not inhibit the cyst wall formation. Interestingly, in non-divided nuclei another round of DNA replication occurred. DNA content of cysts formed in the presence of albendazole, measured by flow cytometry, was equal to untreated cysts (16N).

Nuclear division and cell organization during differentiation

Albendazole induced inhibition of nuclear division in encysting cells indicated the presence of microtubular mitotic spindle; therefore we focused on visualization of the nuclei division. The studies of ultrastructure in encysting cells on electron microscope revealed that division of both nuclei occurs in the early phase of encystation in a precyst. The nuclei divided by semi-open mitosis correspondingly to nuclei division in *Giardia* trophozoites. Two microtubular mitotic spindles were formed; each composed of extranuclear and intranuclear microtubules. The nuclear membrane did not break apart during mitosis and was perforated only for the single microtubules to enter the nucleus at the poles. A basal body was situated at the proximity to the pole, where the microtubules concentrated. Nuclei after mitosis stayed in pairs with parts of plasmatic membranes lying close together - resembling diplokaryons. The adhesive disc was fragmented into pieces composed of microtubules with adjacent microribbons. As revealed with immunofluorescence imaging, phosphorylation including that of aurora kinase, did not accompany the adhesive disc fragmentation during encystation in contrast to disassembly of the disc in trophozoite division. Nuclei during division had a characteristic irregular shape, from the nuclear envelope projected ER. This lobular shape also maintained nuclei in early cysts. In mature cysts with assembled cyst wall, the nuclei were mostly rounded, due to decrease of ER expansion as ESV production ceased in cysts. The nuclei within the pair were interconnected with cytoplasmic bridges. Each nuclear pair was interconnected with up to three small bridges that can vary in size. As only few precysts contained nuclear pairs without the interconnection, we propose that nuclei interconnected shortly after karyokinesis by a two-step fusion, with the outer nuclear envelopes fusing first. These inter-nuclear bridges were present either in precysts, or in cysts of any origin: in *in vitro* formed mature cysts that are resistant to water treatment or even in wild-type cysts gained from animals or human patients.

During the whole encystation only one single flagellar apparatus comprising four pairs of flagella and eight adjacent basal bodies remained preserved. Confocal microscopy using

antibody against acetylated tubulin showed that the arrangement of axonemes was preserved the same as that in interphase trophozoite. The nuclear pairs were situated adjacent to the basal bodies tetrads at one cyst pole. And in each nuclear pair just one nucleus was positioned in close proximity of the basal body mediating the nuclear pair association to basal body. All these observations were supported and confirmed by ultrastructural analysis.

Excystation

Although the excystation of *Giardia* was described in 1980s´ by scanning electron microscopy, up to date basic structural description of freshly excysted trophozoite was missing. Moreover, it is not understood how the nuclear pairs and single flagellar apparatus are distributed into two subsequent cell divisions that follow the excystation. During the excystation the first six backward directed flagella, namely caudal, posteriolateral and ventral emerged from a single opening. The anteriolateral flagella together with nuclei were the last to emerge from the cyst. Its cytoskeleton comprised the single flagellar apparatus and two duplicated microtubular structures as shown with immunostaining. The single flagellar apparatus remained in the same position as in the interphase trophozoite or the cyst. Microtubular arcs corresponding to defragmented adhesive disc were positioned left or right next to backward directed flagella. Trophozoite had one more, so far undescribed, cytoskeletal microtubular structure stained with anti-acetylated tubulin; these were two microtubular bundles lying symmetrically along the caudal flagella. These likely corresponded to median bodies. Both of these microtubular structures (defragmented disc and microtubular bundles) disappeared during cell division. The adhesive discs in newly forming daughter trophozoites in cytokinesis oriented with ventral/ventral axial symmetry were developing in the opposite directions to each other, winding counter clockwise corresponding to typical cytokinesis of trophozoite (Tůmová et al. 2007b). Distribution of parental flagella between daughter trophozoites in the first cell division after excystation was similar to that of mitotic trophozoites (Nohýnková et al. 2006). The anteriolateral and posteriolateral flagellar pairs received progeny from mother trophozoite, while ventral and posteriolateral pairs were assembled *de novo* and were observed as short axonemes on fluorescence microscopy or Giemsa staining in freshly divided cells.

5. DISCUSSION

Aphidicolin influence on cell cycle

Two synchronizing methods for *Giardia* trophozoites have been published and both of them used aphidicolin, either alone (Reiner et al. 2008) or in combination with the microtubule polymerization inhibitor nocodazole (Poxleitner et al. 2008a). In our study, we further tested the effect of aphidicolin on *Giardia* trophozoites in order to evaluate and better understand the consequences of these synchronization methods on individual *Giardia* trophozoites using an extended repertoire of cell biological characteristics. It is widely accepted that aphidicolin inhibits DNA replication in eukaryotic cells and this effect is used for arresting cells in the G1/S phase border (Pedrali-Noy et al. 1979). Using two approaches - BrdU incorporation and flow cytometry analysis - to study DNA replication, we confirmed that DNA synthesis in *Giardia* trophozoites is stopped during aphidicolin treatment. Prolonged stalling of the DNA replication fork as a result of aphidicolin treatment may result in DNA damage and checkpoint activation in HeLa cells (Liu et al. 2003). *Giardia* has two copies of the core histone H2A (Wu et al. 2000, Yee et al. 2007), which is involved in signaling pathway triggered as a reaction to DSBs in mammalian cells. The histone sequences in *Giardia* show clear similarity to histones known from other eukaryotes and near its C-terminus it contains the same SQ motif, which is characteristic for phosphorylation and phosphorylated in the variant H2AX in response to DNA damage (Chambers and Downs 2007). We found that this posttranslational modification of histone H2A is induced in *Giardia* exposed to aphidicolin. Unrepaired or incorrectly repaired DNA lesions or chromosomal aberrations can lead to cancer or cell death (Zhou and Elledge 2000). In response to such DNA disorders like DSB, cells have developed highly sophisticated systems to recognize DSB and arrest cell cycle until the lesions are corrected. *Giardia* cells during replacement of the histone H2A after the release from aphidicolin reenter cell division, which might indicate that DSB are repaired through DNA repair machinery. We suppose that this block of mitotic entry after aphidicolin-mediated DNA damage and following reentry into mitosis after the release from aphidicolin is a strong indication for the presence of a DNA damage checkpoint in *Giardia*. However, further studies are needed to demonstrate whether components of a DNA damage checkpoint signaling cascade are activated in *Giardia* in response to exposure to aphidicolin.

While DNA replication and entry into mitosis in aphidicolin treated *Giardia* trophozoites stopped as discussed above, other cell cycle processes continue. Increased cell size together

with significantly higher protein content in treated cells, suggests that the nuclear and cytoplasmic cycles are dissociated. Increasing cell size has already been documented in sea urchin embryos (Sluder and Lewis 1987), *Paramecium caudatum* or bloodstream forms of *Trypanosoma brucei* under aphidicolin influence (Sabaneyeva et al. 1999, Mutomba and Wang 1996) or in senescent cells in mammalian cell cultures (Campisi et al. 1996). Therefore, it seems that cells that cannot enter mitosis for some reason (activated checkpoint due to the presence of inhibitor, lack of nutrients, senescent cells) continue to synthesize proteins and increase in cell size.

Nuclear division and cell organization during differentiation

Nuclear division during *Giardia* differentiation is one of the poorly understood aspects of the life cycle of this pathogen. It is well known that the *Giardia* trophozoite is bi-nucleated whereas the cyst is quadri-nucleated. However, karyokinesis during encystment has not been documented so far. In the trophozoite, division of nuclei is tightly coupled to the cell division, i.e., to the mitotic reorganization of cytoskeleton including complicated duplication of flagellar apparatus, and cytokinesis (Nohýnková et al. 2006; Tůmová et al. 2007b). In the precyst, nuclear division is independent of cell division. Nuclei divide in a non-dividing cell with a single flagellar apparatus, which corresponds to that of an interphase trophozoite; no new basal bodies/axonemes are formed and no cleavage furrowing is induced. Despite nuclear division in precyst, the topological arrangements of mastigonts of the cyst with four nuclei correspond to that in trophozoites (Sheffield and Bjorvatn 1977). The two karyomastigonts are linked to pertinent tetrad of basal bodies positioned side by side between the nuclei. Instead of two nuclei in trophozoite, there are two pairs of interconnected nuclei in cyst. And only one nucleus from each pair is positioned in close proximity to relevant tetrad of basal bodies. We found that the interconnection of nuclei by fusion of both nuclear membranes in *Giardia* cyst is not a temporary or occasional event occurring only in one nuclear pair during the encystation or within already formed cyst. We present data showing that this interconnection of nuclear membranes is present either in precysts, or in cysts of any origin: in *in vitro* formed mature cysts that are resistant to water treatment or even in wild-type cysts gained from animals or human patients.

Nuclei interconnection in cyst stage was shown on electron micrographs also earlier; however authors described this process differently: either as an unfinished nuclear division in cyst (Solari et al. 2003) or as an unfinished nuclear division during excystation (Hetsko et al. 1998). Poxleitner et al. (2008b) regards the nuclear fusion in a cyst as a mark of genetic

exchange. Anyway all the authors so far have described this phenomenon as temporary. Fusion of nuclei might function not only for possible genetic recombination as suggested by Poxleitner et al. (2008b) but might serve as mechanical bond within the nuclear pair in cyst and especially during excystation when the karyomastigont has to squeeze through the small opening in cyst wall and stay interconnected as we documented on structural description of the excyzoite, in which the nuclear pairs are still held together.

6. SUMMARY OF RESULTS

Aphidicolin influence on cell cycle

- DNA replication inhibitor aphidicolin causes inhibition of DNA synthesis in *Giardia*: trophozoites are arrested at the G1/S border according to their DNA content. Consequent inhibition of entry into mitosis and cytokinesis indicates that *Giardia* has functioning DNA damage checkpoint.

- Presence of phosphorylated histone H2A, a marker of double strand breaks, suggests that aphidicolin may cause DNA damage in *Giardia*. Reversibility of this posttranslational modification after inhibitor removal indicates that *Giardia* possesses DNA damage reparatory mechanisms.

- Aphidicolin treatment causes dissociation of nuclear and cytoplasmic cycles. While DNA synthesis and entry to mitosis are stopped, the cytoplasmic cycle and its processes continue: increasing cell size, corresponding to higher protein content and higher percentage of cells containing median bodies.

Albendazole influence on cell cycle

- Albendazole inhibits nuclei division in *Giardia* trophozoites by blocking mitotic spindle assembly. Nuclei that did not undergo karyokinesis proceed to the second round of DNA replication. The control mechanism of allowing DNA synthesis only once per cell cycle seems to be missing or circumvented in *Giardia*.

Nuclear division and DNA replication during encystation

- Replication of DNA during *Giardia* encystation occurs after nuclei division. BrdU incorporation revealed that DNA replication occurred in all four nuclei within the cyst.

- Albendazole blocks nuclei division during *Giardia* encystation. However, it does not prevent cyst wall formation – these two processes are not interconnected. The nuclei within cysts formed in the presence of albendazole undergo another round of DNA replication without previous karyokinesis.

Nuclear division and cell organization during differentiation

- Nuclei division occurs in early phase of encystation before cyst wall formation in a precyst. Each nucleus is divided by its own mitotic spindle composed of extranuclear and intranuclear microtubules. Nuclei are divided by semi-open mitosis as in trophozoites.

- Nuclei after the karyokinesis stay in pairs on one cyst pole. Nuclei in pairs are interconnected with several inter-nuclear bridges, formed by fusion of both nuclear membranes. This nuclear organization in cyst persists in mature wild-type, as well in *in vitro*, formed cysts.

- Flagellar apparatus does not replicate during karyokinesis in encystation, which is in contrast to karyokinesis in trophozoites. The arrangement of flagellar apparatus remains same as in interphase trophozoite. In each nuclear pair only one nucleus is associated with one tetrad of basal bodies.

Excystation

- Excyzoite is rounded; four nuclei are still organized in two pairs. The cytoskeleton comprises a single flagellar apparatus, which remains in the same arrangement as in the trophozoite and the cyst. Next to backward directed axonemes are fragments of adhesive disc. Along caudal flagella lie two microtubular bundles which likely correspond to median bodies.

7. REFERENCES

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a) Publications for Ph.D. dissertation

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b) Other publications

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