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Giardia lamblia Under Microscopy – How This Primitive Protist Divides

Marlene Benchimol

Universidade Santa Úrsula, Laboratório de Ultraestrutura Celular, Rio de Janeiro, Brazil

Correspondence: **marleneben@uol.com.br**

ABSTRACT

Giardia lamblia is a parasitic protozoan that infects thousands of people all over the world, causing a disease known as Giardiasis. *Giardia* trophozoites are tear-shaped cells with two nuclei located at the anterior region of the cell body. *Giardia* is an amitochondrial flagellate and possesses a complex cytoskeleton based on several microtubular systems. In the interphase, these microtubules include the axonemes of the eight flagella, the median body and the funis, both formed by a set of microtubules and the ventral adhesive disc built on a helicoidally turned layer of parallel microtubules. There are several questions still open to debate concerning the basic biology of *Giardia* and a fundamental one among these is its process of division. *Giardia* presents a semi-open type of mitosis, in which the nucleus elongates and an extranuclear spindle is formed. Some of the spindle microtubules penetrate through small openings in the nuclear envelope. The mode of *Giardia* cytokinesis and karyokinesis has been discussed in several papers due to the controversy of how the cells maintain their left-right (dorsal-ventral) asymmetry. Recently, progress has been made and it has been shown that *Giardia* divides with mirror-image symmetry. Elegant experiments were performed using a single clone of trophozoites transfected with a plasmid and also the spindle was finally demonstrated by electron microscopy. Although molecular data and the genome project have provided advances in *Giardia* knowledge there are still several questions to be answered concerning *Giardia* division. Thus, this review presents an analysis of the advances made on the knowledge of how *Giardia* divides and the behavior of the two nuclei in interphase and mitosis.

Keywords: division, giardia, karyokinesis, mitosis, spindle

CONTENTS

INTRODUCTION

Giardia lamblia is a parasitic protozoan that infects thousands of people all over the world, causing a disease known as Giardiasis. The trophozoite form of *G. lamblia* has a characteristic pear-shaped body, 12-15 mm long and 5-9 mm wide. It is a bi-nucleated amitochondrial flagellate and possesses a unique cytoskeleton in which the protein tubulin

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Invited Review

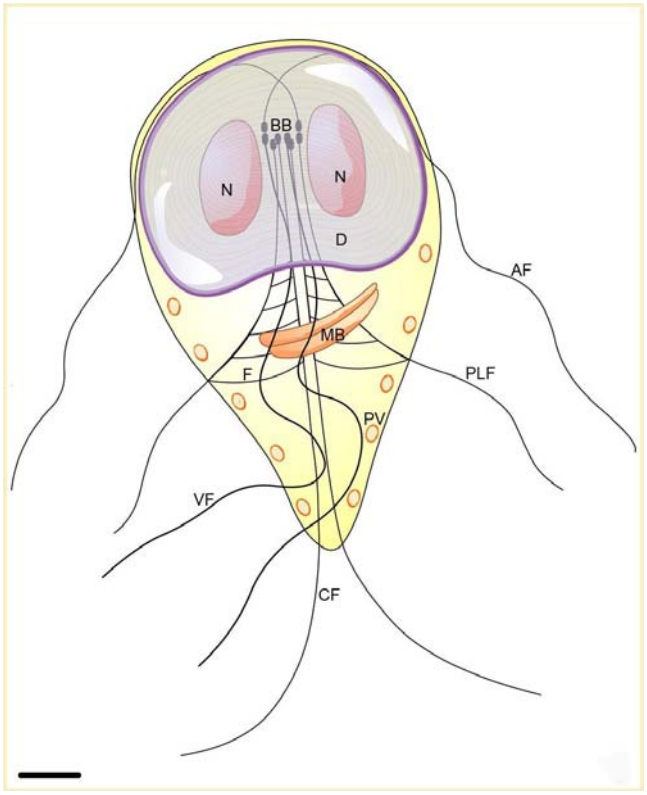

Fig. 1 Schematic diagram showing the arrangements of cell structures in the interphase *Giardi*a. Note the disc (D) situated at the ventral region, the two nuclei (N), the flagellar pairs and basal body pairs (BB). The median body (MB) is seen transversely to the axonemes, and the funis (F) are microtubules connecting the central axonemes to the postero-lateral flagella axonemes. AF, anterior flagella; PLF, posterior-lateral flagella; VF, ventral flagella; CF, caudal flagella; PV, peripheral vesicles. Bar = 1 μm. Benchimol, unpublished.

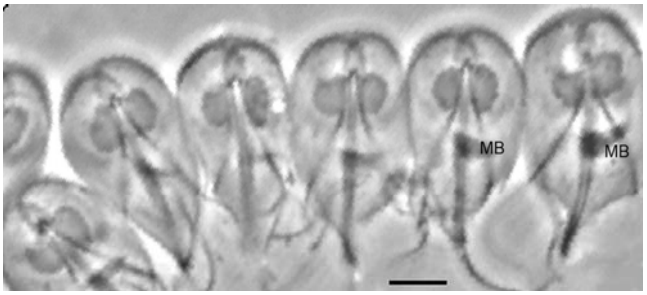

Fig. 2 *G. lamblia* images observed by light microscopy phase contrast. Two nuclei (N), median body (MB) and flagella can be seen, as well as the cell sizes and shapes. Occasionally, condensed chromosomes can be depicted. Bar = 2 μm. Benchimol, unpublished.

predominates in the following structures: four pairs of flagella (**Figs. 1-4A**), an adhesive disc (**Figs. 1, 4-6B, 9**) composed of microtubules and microribbons containing giardins, a median body (**Figs. 1, 7**) (Piva and Benchimol 2004) and a funis (**Figs. 6B, 9**) made up of sheets of microtubules following the axonemes from the caudal flagella (Adam 2001; Benchimol *et al*. 2004). The point of origin of the basal bodies of the eight flagella lies deep in the cell body (**Fig. 1**). The ventral disc is composed by a single layer of 25 nm microtubules, forming an asymmetrical spiral, which is very distinct in *G. lamblia* and *G. muris*, but it is not as distinct in *G. agilis*. The median body of *G. lamblia* can be used as a taxonomic criteria, since this microtubular structure differs in position and shape in *G. agilis*, *G. lamblia* and *G. muris* (Filice 1952). Its size varies between 0.2 μm and 1.8 μm (average of 0.84 μm) in width and 0.8 μm to 8.0 μm (average of 3.34 μm) in length (Piva and Benchimol 2004).

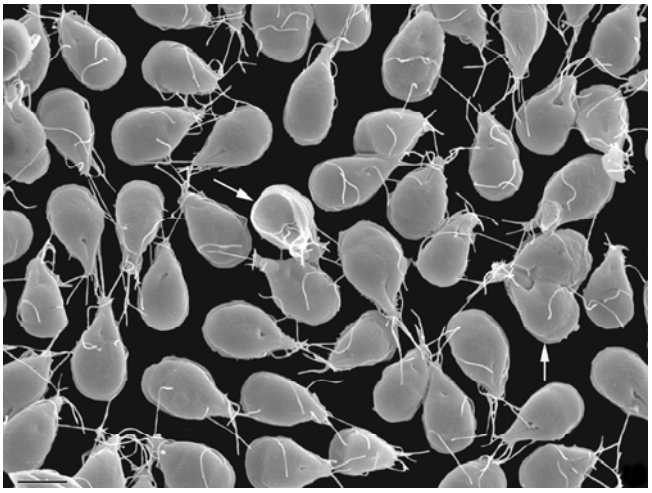

Fig. 3 Low magnification of several *Giardia lamblia* adhered by the ventral region as seen in scanning electron microscopy. Arrows point to dividing cells. Bar = 5 μm. From Benchimol (2004c) *Protist* **155**, 33-44, with kind permission from Elsevier.

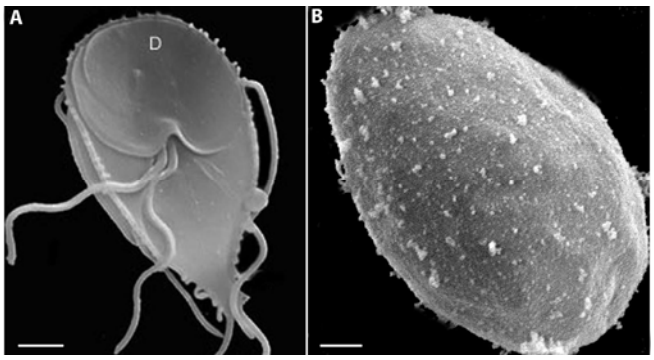

Fig. 4 Scanning electron microscopy of *G. lamblia* in a trophozoite form (a) and as a cyst (b). D, disc. Bar = 1 μm. **Fig. 4a** from Benchimol (2004c) *Protist* **155**, 33-44, with kind permission from Elsevier; **Fig. 4b**, Benchimol, unpublished.

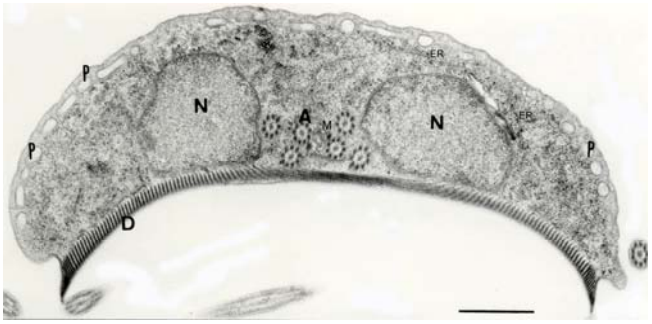

Fig. 5 TEM routine preparation of *Giardia* showing the two nuclei (N), the ventral disc (D), peripheral vesicles (P), the flagellar axonemes (A) and the mitosomes (M). The endoplasmic reticulum (ER) is distributed all over the cytoplasm. The proximity of axonemes and nuclei are noted. Bar = 1 μm. From Benchimol *et al.* (2004) *Journal of Structural Biology* **147**, 102-115, with kind permission from Elsevier.

The trophozoite form of this protist lacks organelles found in higher eukaryotes, such as a traditional mitochondria and peroxisomes (Gillin *et al*. 1996), although Tovar *et al*. (2003) demonstrated that *Giardia* contains mitochondrial remnant organelles (mitosomes) bounded by double membranes that function in iron-sulfur protein maturation. This finding indicates that *Giardia* is not primitively amitochondrial and that it has retained a functional organelle derived from the original mitochondrial endosymbiont. Mitosomes are localized in small cellular structures distributed through-

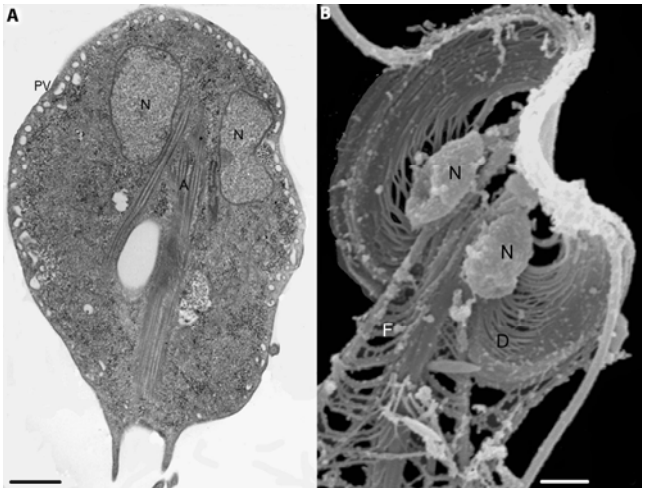

Fig. 6 *Giardia* trophozoites observed by TEM routine preparation in a longitudinal section (A) and after membrane extraction by detergents (B) observed by SEM. The two nuclei (N) and the flagellar axonemes (A) are seen in both figures. The ventral disc (D) and the funis are better visualized in **B**, whereas the peripheral vesicles (PV) are seen in **A**. Bar = 1 μm. **Fig. 6A**, Benchimol, unpublished; **Fig. 6B**, Benchimol (2005) *Archives of Microbiology* **183**, 62-72, with kind permission from Springer Science & Business Media.

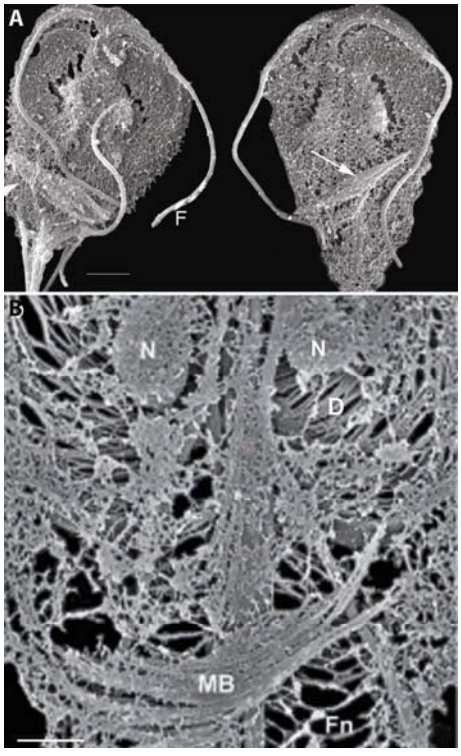

Fig. 7 *Giardia* trophozoites observed by FESEM (Field Emission Scanning Electron Microscopy) after detergent extraction. The plasma membrane was partially removed, allowing observation of the cytoskeleton, the median body included (arrows). The ventral disc (D), the two nuclei (N), median body (MB), and (F) are seen. Every fascicle that constitutes the median body is observed. The fascicles number is variable as well their disposition and location. F, funis. Bars = 1 μm. From Piva and Benchimol (2004) *Biology of the Cell* **96**, 735-746, with kind permission from Elsevier.

out the cytoplasm, including this organelle accumulation around basal bodies (**Fig. 5**).

Structures such as the Golgi complex seem to be absent in trophozoites (Reiner *et al*. 1990; Lujan *et al*. 1995), although this is controversial (Lanfredi-Rangel *et al*. 1999). *Giardia* also presents a system of peripheral vesicles (**Figs. 5, 6A**), which correspond to early and late endosomes and to lysosomes (Lanfredi-Rangel *et al*. 1998). *Giardia* is an intriguing parasite since it has an unusual morphology of bearing two nuclei.

Previous studies have suggested that the peripheral vesicles of *Giardia* may correspond to early and late endosomes and to lysosomes (Lanfredi-Rangel *et al*. 1998). Internal vesicles of unknown function were also described (Benchimol 2002).

There are several questions still open concerning basic biological properties of *Giardia*, and one fundamental question is its process of division, which is still under debate. While studies on molecular aspects of gene organization and expression in *Giardia* have advanced rapidly over recent years, there are few papers dealing with *Giardia* division. Many of which were carried out with light microscopy (Filice 1952; Soloviev 1963; Kabnick and Peattie 1990; Cerva and Nohýnková 1992; Ghosh *et al*. 2001; Yu *et al*. 2002), whereas few papers were published with more details using electron microscopy (Benchimol 2004a, 2004c; Nohýnková *et al*. 2006; Sagolla *et al*. 2006).

Since there are controversial aspects concerning *Giardia* division, this review intends to present an analysis of the advances made on the knowledge of how *Giardia* divides and the behavior of the two nuclei in interphase and mitosis.

The median body

The median body (MB) (**Figs. 1, 2, 7**) is the least defined microtubular structure in the *Giardia* cytoskeleton. The MB has been described in *G. lamblia* as one or two roughly aligned fascicles of microtubules situated transversely to the axonemes (Filice 1952; Kulda and Nohýnková 1995), but recently Piva and Benchimol (2004) found that it is not just one or two structures, but it varies in number, shape and position. The MB is constituted by several small fascicles formed by microtubules forming larger bundles (**Fig. 7**). The bundle number is variable as well the number of microtubules found in each fascicle. The MB can be used as taxonomic criteria since it varies in different *Giardia* species, on the basis of the morphology of this structure, such as *G. duodenalis*, *G. muris*, and *G. agilis* (Filice 1952). Our group studied *G. lamblia* by a technique that allowed the removal of the plasma membrane and observation of the cytoskeletal structures by both routine scanning electron microscopy (SEM) and field emission high resolution SEM of *G. lamblia* (**Fig. 7**). In addition, the article from Piva and Benchimol (2004) presented new observations concerning the median bodies such as (1) they were found both in mitotic (**Fig. 8**) and interphasic trophozoites (**Figs. 2, 7**); (2) they were present in about 80% of the cells; (3) they could be connected either to the plasma membrane, to the adhesive disc or to the caudal flagella, and thus they are not completely free in the cells, as published before; (4) a MB buddle can protrude the cell surface; (5) the MB microtubules react with several anti-tubulin and -β giardin antibodies.

Funis

The funis is made of short arrays of microtubules emanating from the axonemes of the caudal flagella (**Figs. 6B, 9**), which are anchored to dense rods that run parallel to the posterior-lateral flagella (Benchimol *et al*. 2004). Our group showed that after emergence of the posterior-lateral flagella, funis microtubules are anchored to the epiplasm, a fibrous layer that underlies the portion of membrane where tail contractility occurs. Thus, our group proposed a model for the tail flexion of *G. lamblia* in which the funis participates.

The Giardia nuclei

Although molecular data and the genome project have progressed, mitosis in *Giardia* is not fully understood. In order to understand mitosis in Giardia, first it is necessary to understand the behavior of the interphasic nuclei. The

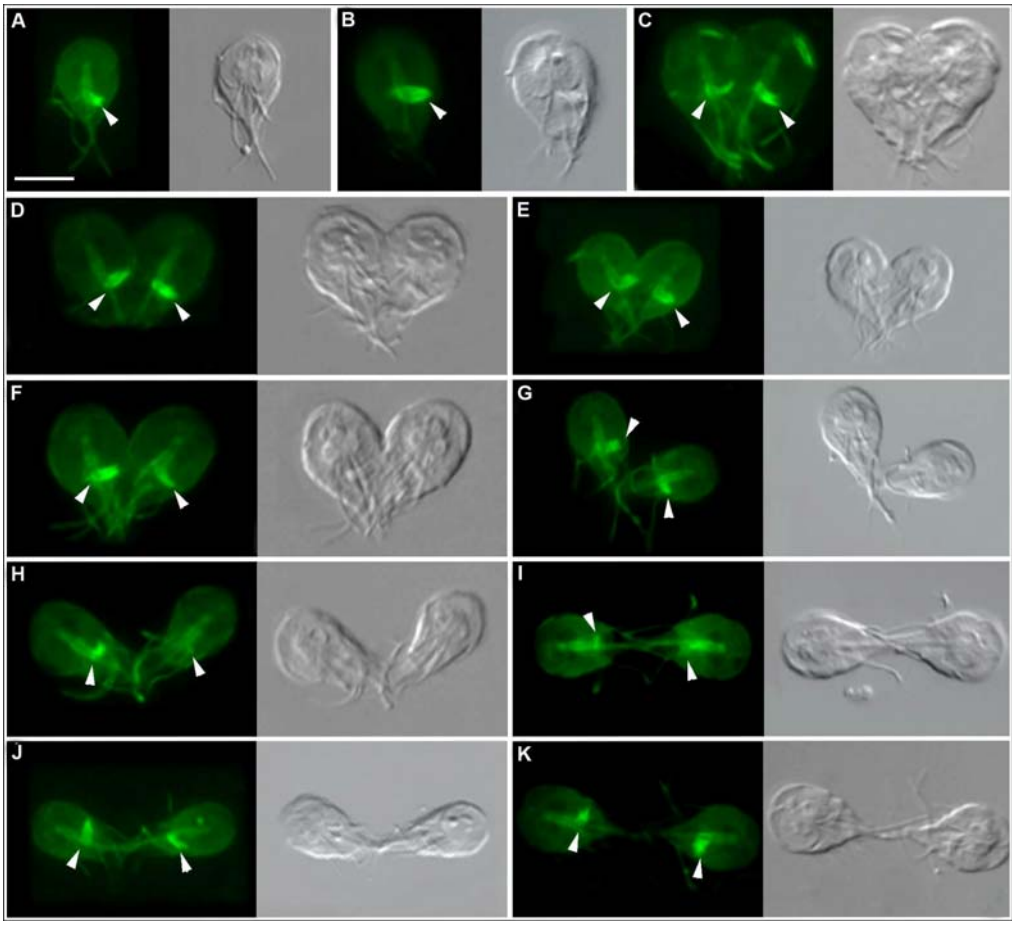

Fig. 8 Persistence of the median body (MB) during *Giardia* mitosis. Localization of tubulin in *Giardia* by immunofluorescence microscopy after staining with monoclonal anti-tubulin antibody with the purpose to label the MB. Cells are seen from interphase (row **A**), to different progressive phases of mitosis (**B-K**) in immunofluorescence and DIC. Labeling is observed in the median bodies (arrowheads), axonemes, flagella and disc, in all phases of the cell division. Note the mirror-symmetry of the median bodies in the late mitosis phase. **H-K**, bar = 5 µm. From Piva and Benchimol (2004) *Biology of the Cell* **96**, 735-746, with kind permission from Elsevier.

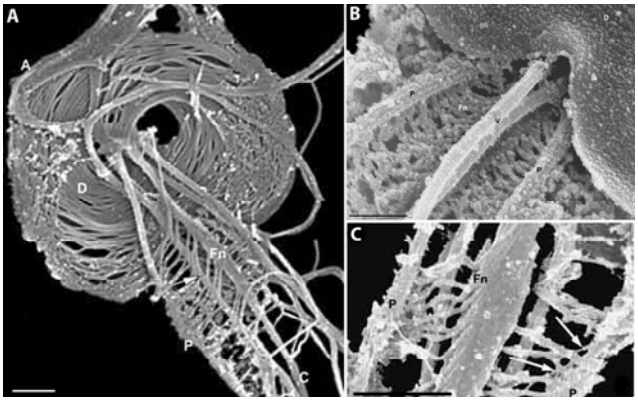

Fig. 9 Field emission scanning electron microscopy (FESEM) after extraction with Triton X-100. The funis (Fn) is observed as microtubules connecting the axonemes of the caudal flagella (C) to the posterior-lateral flagella (P).The adhesive disc (D) and the anterior (A), posterior-lateral (P) and caudal flagella axonemes (C) are seen. The caudal region shows the funis microtubules (Fn) emanating from the caudal flagella axoneme (C) to the posterior-lateral flagella (P). In **Figs. 9B, 9C**, a close view of the funis microtubules (Fn) fanning out towards the posterior-lateral flagella (P). Note that they are anchored to the dense rods (arrows). Bars = 1 µm. From Benchimol *et al.* (2004) *Journal of Structural Biology* **147**, 102-115, with kind permission from Elsevier.

nuclei of trophozoites has been studied using fluorescence microscopy (**Fig. 10**), field emission scanning electron microscopy (**Fig. 7B**), routine scanning (**Fig. 6B**) and transmission electron microscopy (**Figs. 5, 6A**), freeze-fracture (**Figs. 11-13**), immunocytochemistry, and 3-D reconstruction (Solari *et al*. 2003; Benchimol 2004a, 2004b, 2004c, 2005; Nohýnková *et al*. 2006; Sagolla *et al*. 2006).

The two nuclei present slight differences

Of all the cellular features within the trophozoite, the most puzzling and intriguing is the presence of two nuclei without nucleoli which are bilaterally symmetrical (**Figs. 1, 2, 5-7**). *Giardia* has two diploid nuclei (2n=10), which are both transcriptionally active and identical in DNA content (Wiesehahn *et al*. 1984; Kabnick and Peattie 1990; Yu *et al*. 2002). Previous studies have suggested that these nuclei are equal in size and it has also been demonstrated that the DNA in each nucleus contains sequences specifying the ribosomal RNA (Kabnick and Peattie 1990). This last group also demonstrated that the DNA in each nucleus contains sequences specifying the ribosomal RNA. They suggested that the DNA in both nuclei is functionally equivalent and equally likely to serve as the template for the ribosomal RNA that makes its way out of the nucleus and into the cytoplasm.

Nuclear envelope

Morphological studies using different techniques (Benchimol 2004b) suggested that the two nuclei in *Giardia* are not equal since (1) both nuclei in the same cell are distinct in nuclear pore number and distribution (**Figs. 11-12**); (2) nuclear pore complexes are frequently clustered in nuclear envelope domains; (3) dividing nuclei display very few nuclear pores (**Fig. 13**); (4) few ribosomes are found in the outer nuclear envelope of the trophozoite (**Fig. 14A**).

HOW TO OBTAIN MITOTIC GIARDIA?

Several procedures have been used in order to improve the number of mitotic cells.

Benchimol method

Cell cultures were enriched for mitotic cells by growing cells routinely until confluency (48-72 h). Fresh, warmed

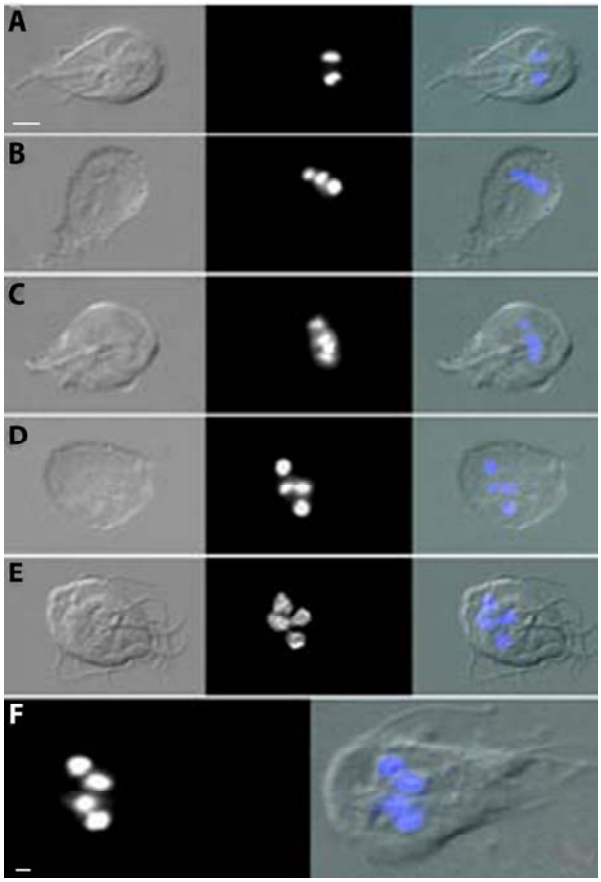

Fig. 10 *G lamblia* images by DIC (first row), fluorescence microscopy after DAPI staining (middle row) and in overlay (third row). In (**A**) two nuclei (N) are observed in an interphasic cell. Note that one nucleus divides slightly out of phase and thus a cell presenting three nuclei can be observed (**B, C**). In **D**, four nuclei are observed. Notice that they are taking different positions when compared to interphasic nuclei, as in **A**. The nuclei move and finally a cell with four nuclei is seen (**E, F**). Bars = 1 μm. From Benchimol (2004b) *Parasitology Research* **94**, 254-264, with kind permission from Springer Science & Business Media.

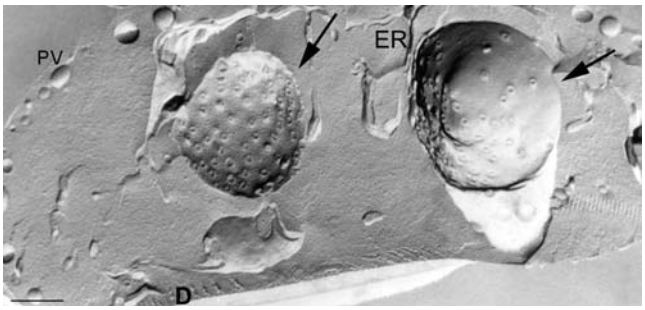

Fig. 11 Freeze-fracture of *G lamblia* showing the two nuclei. Notice that the nuclear pore numbers and distribution are quite distinct (arrows). D, disc; PV, peripheral vesicles, ER, endoplasmic reticulum. Bar = 1 μm. From Benchimol (2005) *Archives of Microbiology* **183**, 62-72, with kind permission from Springer Science & Business Media.

medium was added to the tube and cultures were incubated at 37°C. Cells from the supernatant were collected at one-hour intervals for 10 h in order to collect the mitotic cells.

Nohýnková method (Nohýnková *et al*. 2006)

A cultured population was enriched with mitotic cells according to a protocol based on the specific effect of albendazole on *Giardia* cells. Mid-log-phase trophozoites nearly forming a confluent monolayer were exposed to 100 ng of albendazole (Sigma)/ml in fresh TYI-S-33 medium for 7 h.

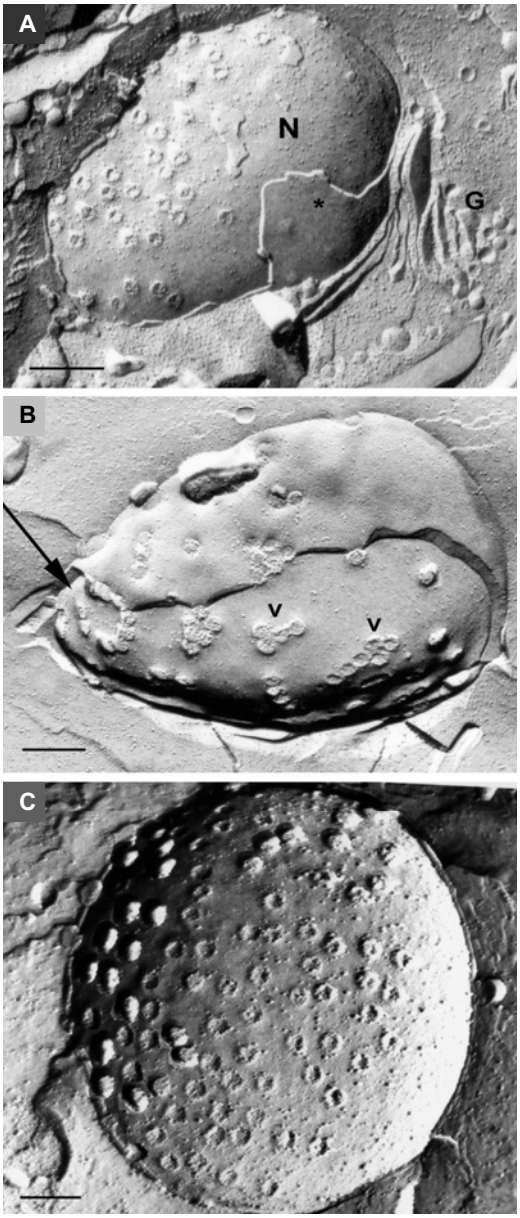

Fig. 12 Freeze-fracture of *G lamblia* showing the diversity of distribution of the nuclear pores in the nuclei (N). Notice that in **A** the stacked lamellae is similar to the Golgi complex (G) and a membranous profile envelopes the nucleus (asterisk). In **B** the nuclear pores are clustered (arrowheads), whereas in **C** a high number of nuclear pores are homogeneously distributed. Bar = 1 μm. From Benchimol (2004b) *Parasitology Research* **94**, 254-264, with kind permission from Springer Science & Business Media.

The culture medium plus free-swimming albendazole-affected cells were then quickly discarded and the adherent cells were immediately overlaid with the fresh medium without drug. The cultures were chilled on an ice-water bath for 5 min to detach the cells. The suspension was immediately injected into a 3-mm-deep perfusion anaerobic chamber (Sigma), followed by incubation at 37°C for 10 min. During this period the cells attached and entered mitosis.

Sagolla method (Sagolla *et al*. 2006)

Cell cultures were enriched for mitotic cells by growing for 1-2 days past confluency. Fresh, warmed medium was added to the tube and cultures were incubated at 37°C for 3-7 hours. Cells were collected at one-hour intervals in order to collect the maximum number of mitotic cells. Generally 10-20% mitotic cells were observed in each culture.

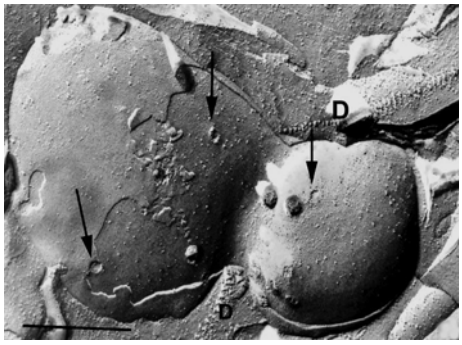

Fig. 13 Freeze-fracture of a dividing nucleus in *G. lamblia* trophozoites. Note that the nuclear envelope displays few nuclear pores (arrows) and the disc (D) contacts the region of karyokinesis. Bar = 1 μm. From Benchimol (2004c) *Protist* **155**, 33-44, with kind permission from Elsevier.

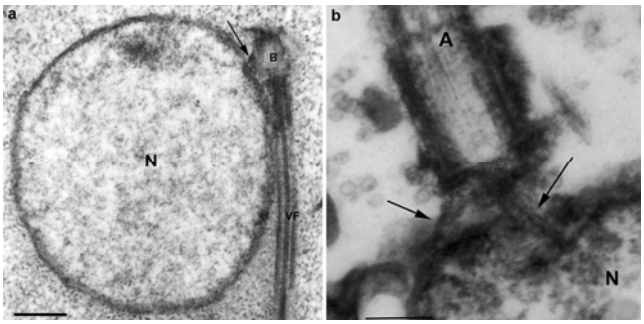

Fig. 14 Routine preparation for transmission electron microscopy of *G. lamblia* showing one of the ventral flagellum axoneme (VF) in close contact with the nucleus (N). The arrow points to a connection between the basal body (B) and the nuclear envelope. Bar = 400 nm. **Fig. 14B**: a high magnification allows the observation of links (arrows) connecting the nuclear envelope to the axoneme (A). Bar = 200 nm. From Benchimol (2005) *Archives of Microbiology* **183**, 62-72, with kind permission from Springer Science & Business Media.

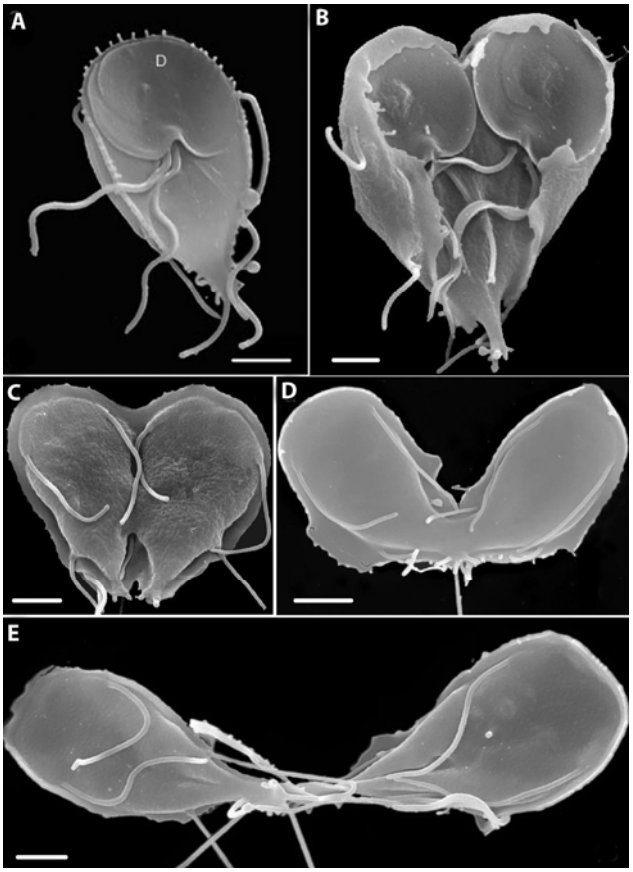

Fig. 15 General views of some interphasic steps (A) and dividing *Giardia* (B-E). Bar = 1 μm. From Benchimol (2004c) *Protist* **155**, 33-44, with kind permission from Elsevier.

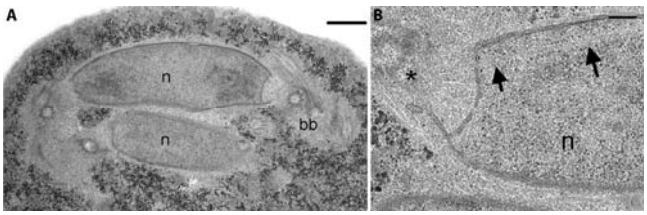

Fig. 16 As shown by TEM, the mitotic spindle is semi-open and basal bodies with axonemes are present at the four spindle poles. (**A**) Cross-section of a mitotic nucleus; microtubules form a sheath surrounding the nucleus. The nuclear envelope is intact except for large openings at the poles. Bar = 200 nm. (**B**) High-magnification view of spindle pole. Cytoplasmic microtubules enter the nucleus through a large opening in the nuclear membrane (arrow). Spindle microtubules (*) radiate from the basal body seen in cross-section, which organizes the spindle microtubules. bb, basal body; n, nucleus. Bar = 50 nm. Micrographs kindly provided by Joel Mancuso and W. Zacheus Cande from University of California, Berkeley.

Techniques used to better understand *Giardia* division

Several methods have been used in order to better understand this unusual cell. Since *Giardia* has two nuclei and a complex cytoskeleton complementary techniques were used by different groups. Filice (1952), and Cerva and Nohýnková (1992) worked using light microscopic of stained samples. Erlandsen and Rasch (1994) investigated the DNA content using the microdensitometric quantitation of Feulgen staining by confocal microscopy. Nohýnková *et al*. (2000) also used immunofluorescence microscopy and anti-tubulin antibodies. Ghosh *et al*. (2001) and Yu *et al*. (2002) used nuclear stains, confocal microscopy, anti-tubulin antibodies and plasmid transfection into a single nucleus in order to follow how the two nuclei are partitioned during mitosis. Solari *et al*. (2003) used serial thin-sectioning, observation on TEM and further 3D-reconstruction and Benchimol (2004a), who additionally, used freeze-fracture (**Figs. 11-13**), immunolabeling and a technique in which the plasma membrane was removed (**Figs. 6B, 7, 8**) allowing observation of the cell interior by scanning electron microscopy, both conventional SEM and field emission SEM (FESEM). Recently, Sagolla *et al*. (2006) in order to test hypotheses of both the mode and mechanism of mitosis, imaged mitotic cells in 3D with conserved molecular markers of the mitotic spindle (anti-tubulin), the centromere (cenH3::GFP), and the basal bodies and the spindle poles (anti-centrin). In addition, FISH of an episomal plasmid to track the fate of either nucleus during cell division has been used by some authors (Ghosh *et al*. 2001; Yu *et al*. 2002; Sagolla *et al*. 2006). All papers have made important contributions to the mitotic *Giardia* puzzle.

Nuclei anchor

An approach using plasma membrane extraction allowed observation of the two nuclei still attached in their original positions (Benchimol 2005). It was shown that nuclei are anchored to basal bodies of the anterior flagella and to the descending posterior-lateral and ventral flagella (**Fig. 14**). This attachment takes place by means of proteinaceous links (**Fig. 14B**).These links were labeled by anti-actin and anti-centrin but not by anti-dynein or anti-tubulin antibodies (Benchimol 2005). In addition, in this paper, fibrillar connections between the nuclei and the disc were demonstrated (**Fig. 14**) and the nuclei exhibited a pendular movement when living cells were treated with cytochalasin, al-

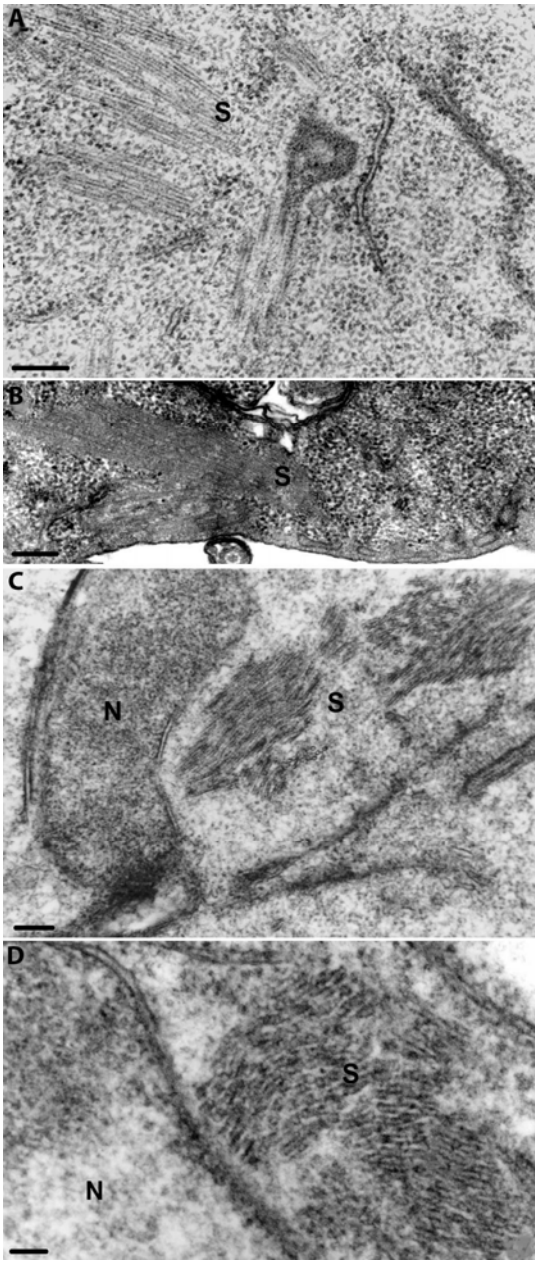

Fig. 17 Views of *Giardia* spindle by transmission electron microscopy. In (**A**) it is possible to note the spindle microtubules irradiating from a basal pole that presents a dense coat. (**B**) shows a longitudinal section of the spindle, showing some parallel microtubules. (**C, D**) show the spindle microtubules in close contact with the nuclear envelope, which presents small openings. Bars = 100 nm. Benchimol, unpublished.

though the nuclei were still connected by their anterior region.

Solari *et al*. (2003) described that the basal body of the anterior flagella is seated perpendicular to the nuclear surface, leaving a narrow (about 50 nm) space between the body and the nuclear envelope, which is filled with a medium-dense material containing fibrils. These analyzes indicated that the nuclei have a defined position and fibrils perform an anchoring system (**Fig. 14**). This raised the possibility of a mechanism for nuclei fidelity migration during mitosis.

Mitosis in *Giardia*

Giardia divides by binary fission (**Fig. 15**). However, because of the presence of two nuclei the steps of *Giardia* mitosis have been difficult to elucidate. Mitosis has been classified as open, semi-open and closed depending whether the nuclear envelope fragments or not during division.

The spindle

The existence of a spindle in *Giardia* has been controversial (Solari *et al*. 2002; Benchimol *et al*. 2004a), since it was difficult to ascertain if the fibers observed by TEM were concerned to spindle microtubules or to other microtubular systems, such as the median body, and funis. Nohýnková *et al*. (2000) showed the spindle by immunofluorescence, but spindle details were not demonstrated. Only a few prior studies sought to identify the stages of mitosis using primarily light microscopy and chromatin staining, yet none described a mitotic spindle (Cèrva and Nohynkova 1992; Filice 1952). It is interesting that this hypothesis was proposed without electron microscopy observations, based on findings by Brugerolle (1974) in another closely related diplomonad *Hexamita*. Only recently, a group (Sagolla *et al*. 2006) has demonstrated dividing *Giardia* by fluorescence microscopy and TEM (**Figs. 16-17**) showing unequivocally the presence of *Giardia* spindles. This group showed two extranuclear spindles that access chromatin through polar openings in the nuclear envelope (**Fig. 16**). However, until now the kinetochore-spindle microtubule associations have not been observed.

Origin of the spindle microtubules

Although Sagolla *et al*. (2006) observed both basal bodies as well as centrin localization of each of the four spindle poles; the origin of the spindle microtubules is still an open question. This group described that each spindle pole was associated with at least one axoneme and that spindle microtubules radiated from one of the basal bodies (**Fig. 17A**). However, the basal body cross-section revealed a ring of doublet microtubules that lack a central microtubule pair, resembling the transition zone of a basal body. It is difficult to establish (1) if this is a pre-existing basal body (2) if it is a new structure that appears during the division and (3) to which flagella axoneme it belongs. In addition, immunolabeling at the electron microscopy level was not performed, consequently it was not possible to clarify whether centrin is localized at the spindle poles.

Behavior of nuclear envelope during mitosis

All groups agree that in *Giardia* the nuclear envelope is present throughout mitosis (**Figs. 16-17**; Nohýnková *et al*. 2000; Benchimol 2004b; Sagolla *et al*. 2006). Sagolla *et al*. (2006) demonstrated that the nuclear envelope elongates with the spindle in anaphase B and openings in the nuclear envelope are seen at the nuclei poles (**Fig. 16**). Spindle microtubules were seen crossing through openings in these nuclear membrane poles, allowing some spindle microtubules to enter the nucleus. This type of mitosis is classified as semi-open.

Semi-open mitosis in *Giardia*

Nohýnková *et al*. (2000) raised the possibility of a semi-open mitosis in *Giardia*. They also showed the spindle by immunofluorescence, but without details. Recently, Sagolla *et al*. (2006) used three-dimensional deconvolution microscopy of each stage of mitosis to monitor the spatial relationships of conserved cytological markers to the mitotic spindles, the centromeres and the spindle poles. In addition, the authors used both light and transmission electron microscopy, and thus determined that *Giardia* has a semi-open mitosis. The persistence of the nuclear envelope throughout mitosis precludes mixing of the chromatin between nuclei. Benchimol (unpublished) also observed an extranuclear spindle represented by microtubules in close contact with the nuclear envelope (**Figs. 17A, 17B**), which present small openings.

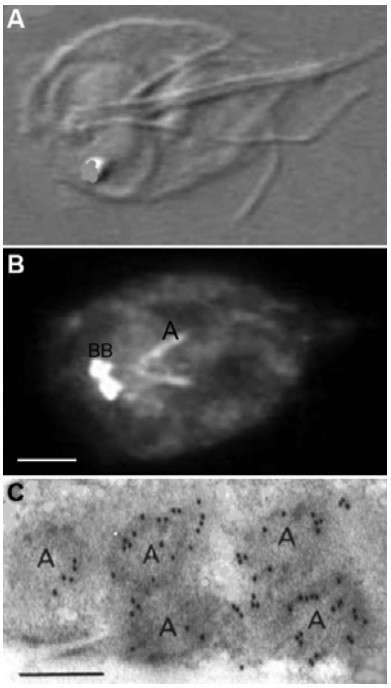

Fig. 18 Immunostaining using anti-centrin antibody. *Giardia* in DIC (**A**), immunofluorescence (**B**), and immunolabeling (**C**). Notice the intense labeling in basal body region (BB), and flagella axonemes (A). Bars: **A, B** = 5 μm; **C** = 300 nm. From Correa *et al*. (2004) *FEMS Microbiology Letters* **233**, 91-96, with kind permission from Elsevier.

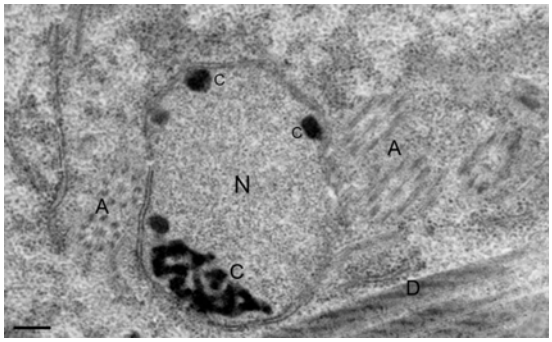

Fig. 19 *Giardia* mitotic prophase. Note that the nucleus (N) presents chromatin condensation (C). A, axonemes; D, disc. Bar = 200 nm. Benchimol, unpublished.

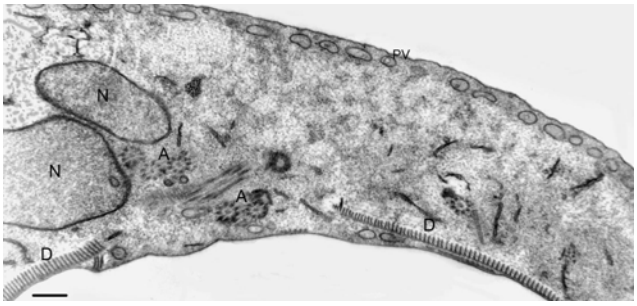

Fig. 20 TEM of a dividing *Giardia* showing that in prophase the nuclei (N) migrate to the cell midline. Note that the axonemes (A) position has changed when compared with interphasic cell (see **Fig. 5**). D, disc; PV, peripheral vesicles. Bar = 300 nm. Benchimol, unpublished.

Drugs affecting the cell cycle

Nohýnková *et al*. (2000) used albendazole in order to obtain a large number of mitotic cells. However, drug treatments are known to affect cell properties, as shown when taxol was used. This drug treatment resulted in lagging chromosomes and half-spindles (Sagolla *et al*. 2006). Thus, drugs should be avoided when studying mitotic events.

Interphase

Interphasic *Giardia* trophozoites present a microtubular cytoskeleton formed by the ventral disc, eight flagellar axonemes with basal bodies, the funis and the median body. During interphase, chromatin is decondensed and the nucleolus is not visible (**Figs. 5, 6A**). In addition, centrin immunolocalization (**Fig. 18**) was found in microtubular structures such as the basal bodies, all the flagella axonemes, in posterior-lateral rods, the adhesive disc, funis and the median bodies (Correa *et al*. 2004). However, Sagolla *et al*. (2006) observed positive centrin staining by immunofluorescence in two clusters between the two nuclei, which co-localized with the flagellar basal bodies.

Mitotic phases

The main transformations that occur in *Giardia* mitosis are presented in **Table 1**.

Giardia mitotic prophase is characterized by chromatin condensation (**Fig. 19**) and the nucleation of spindle microtubules (**Figs 16, 17**). In prophase, each chromosome contained a single centromeric locus. Sagolla *et al*. (2006) showed that in prophase, the nuclei migrate to the cell midline (**Fig. 20**) and that nuclear division occurs along the left-right axis (**Fig. 10**). The two nuclei remain separate during this time (**Figs. 10, 16, 20**) and probably the nuclei do not exchange genetic information. Following nuclear migration to the cell midline, the left-right (L-R) axis of nuclear division yields two daughter nuclei, one from each parental nucleus located on opposite (L-R) sides of the cell (Sagolla *et al*. 2006).

Nohýnková *et al*. (2000) stated that during condensation of chromosomes, seen as clusters within intact nuclei in early prophase, four dots became slightly separated from each other and their fluorescence was reduced. The authors claimed that the arrangement of the dots was in accordance with lateral movement of the two parent kinetosomal tetrads in dividing Giardia occurring at an early phase of mitosis.

Lateral chromosome segregation was interred by fluorescent dots migrating to the nuclei periphery in anaphase (**Fig. 10**). The Sagolla group (2006) stated that the nuclei remain physically and genetically distinct from one another with daughter cells inheriting one copy of both nuclei.

Karyokinesis and disc participation

Evidence has been presented showing the participation of the disc in the karyokinesis (**Fig. 21**) (Solari *et al*. 2002; Benchimol 2004c). Other groups (Ghosh *et al*. 2001; Yu *et al*. 2002) discussed if the two nuclei are partitioned equationally during mitosis and both agree that the division is equational rather than reductional. It means that each daughter cell receives one complete copy of the parental nuclei. In addition, Ghosh *et al*. (2001) suggested that perinuclear tethers are present surrounding the nuclei and thus these structures could be a good candidate for nuclei orienttation during mitosis. Probably these tethers correspond to spindle microtubules that surround the nuclei described in the Sagolla paper (2006). The observation that the two nuclei divide slightly out of phase was first observed by Wiesehahn *et al*. (1984). Recently, it was confirmed and in addition it was demonstrated that the disc participates in the karyokinesis (Benchimol 2004a). The DNA content of trophozoites and cysts of G. *lamblia* using Feulgen staining revealed five chromosomes-like bodies within each nucleus (Erlandsen and Rasch 1994).

Cytokinesis

The *Giardia* division plane has been under intense debate, since there is a disagreement in whether the division is mirror-like (ventral-ventral) or ventral-dorsal (Filice 1952; Ghosh *et al*. 2001; Yu *et al*. 2002). In addition, Benchimol

Table 1 Main cell structures in *Giardia lamblia* trophozoites.

Structure	Location	Characteristics	Function	Open questions
Disc	Ventral region	Spiral array of microtubules and microribons; Comb-like structure	(1) substrate adhesion (2) participation in karyokinesis[7]	Disc morphogenesis
Two nuclei	In the anterior region of the cell	Transcriptionally equivalent diploid nuclei (2n=10) [1] Slight morphological differences[10]	Genetic; division, etc	If meiosis occurs[11]. How the two nuclei divide
Flagella	Anterior; posterior-lateral; ventral; caudal	Eight flagella in four pairs Typical axonemes 9+ 2 arrangement of microtubules	Different cell movements [5, 12]	How they disappear during encystation
Median body	Situated transversely to the axonemes	Several roughly aligned fascicles of microtubules[9]	unknown	Function; behavior during encystation
Funis	Posterior region of the cell body	Of short arrays of microtubules emanating from the axonemes of the caudal flagella to dense rods of the posterior-lateral flagella[8]	Participation on caudal movement (?)	Function; behavior during encystation
Peripheral vesicles	Periphery of the cell	Numerous vacuoles, 200-400 μm	Endosomal/lysosomal[3]	Other functions? Participation in the encysting process?
Mitosomes[6]	Distributed throughout the cytoplasm, and around basal bodies and the base of flagellar axonemes	Small cellular structures bounded by double membranes	Function in iron–sulphur protein maturation	Mitochondrial remnant organelles ?
Golgi complex[2, 4]	Under debate	Under debate	Under debate	If *Giardia* has Golgi
Endoplasmic reticulum	Around the nuclei; dispersed in cytoplasm	Membranous profiles, rough or/and smooth	Participation on protein synthesis; participation on the encystation process	Other functions
Peroxisomes	Unidentified or absent structures	Unidentified or absent structures	Unidentified or absent structures	Which structure performs the peroxisome functions?
Nucleoli	Unidentified or absent structures	Unidentified or absent structures	How ribosomes are formed	How ribosomes are formed

References: (1) Kabnick and Peatie, 1990; (2) Reiner *et al* 1990; (3) Lanfredi-Rangel *et al*. 1998; (4) Lanfredi-Rangel *et al*. 1999; (5) Campanati *et al*. 2002; (6) Tovar *et al*. (2003); (7) Benchimol 2004a; (8) Benchimol *et al*. (2004); (9) Piva and Benchimol (2004); (10) Benchimol 2005; (11) Ramesh *et al*. 2005; (12) Nohynkova´ *et al*. (2006).

(2004c) has suggested multiple modes of cytokinesis in *Giardia*, which could be dorsal-dorsal (**Fig. 22**), ventral-ventral (**Fig. 23**) or along the dorsal-ventral axis, whereas Sagolla *et al*. (2006) stated that it occurs only along the longitudinal axis perpendicular to the axis of nuclear division. This group infers that the longitudinal plane of cytokinesis would favor the inheritance of nuclei with mirror image symmetry in the daughter cells (Sagolla *et al*. 2006).

Equational division model

An equational division process in *Giardia* (Yu *et al*. 2002) has been proposed. In the equational division with ventral-dorsal cytokinesis each daughter cell receives one copy of each nucleus and thus maintains nuclear asymmetry and handedness in the progeny. Equational division with ventral-ventral cytokinesis reverses the handedness with each round of replication. Reductional division with either ventral-dorsal or ventral-ventral cytokinesis homogenizes the nuclei with each round of replication since two left nuclei are segregated to one daughter trophozoite and two right nuclei to the other. Essentially all of the reports agree on equational division model. Thus, nuclei linkage to basal bodies and axonemes would provide nuclear positioning and anchorage. The fact that the nuclear envelope remains intact throughout mitosis could implicate the envelope as an important site of a nuclear anchoring system. The nuclei would move away in concert with the basal bodies setting the correct spatial positioning of nuclei during mitosis.

Benchimol (2004c) proposed that *Giardia* divides with mirror-image symmetry when the division is ventral-ventral (**Fig. 24**) or dorsal-dorsal. Although it has been proposed that *Giardia* divides along multiple division planes depending on whether the cells are attached or unattached to a substrate (Benchimol 2004c), this was not in agreement with the Sagolla group (2006). This group examined populations of cells that were both attached and unattached at the time of fixation and determined that chromosome segregation only occurs along the left-right axis of the cell with cytokinesis perpendicular to that plane. This group also claimed that they never detected cytokinesis occurring in the dorsal-ventral plane as Benchimol (2004c) had found.

Giardia divides with mirror-image symmetry

The question how *Giardia* divides is discussed among several authors (Ghosh *et al*. 2001; Yu *et al*. 2002; Solari *et al*. 2003; Benchimol 2004c; Sagolla *et al*. 2006). However, one point in accordance is that this protist divides with mirror-image symmetry. Ghosh *et al*. (2001) stated that the division occurred in the plane of the adherence disc, so that the right nucleus of the mother becomes the left nucleus of the daughter-cell. Sagolla *et al*. (2006) proposed a nuclear migration model of mitosis with lateral chromosome segregation along the left-right axis and cytokinesis along the longitudinal plane (perpendicular to the spindles), ensuring that each daughter inherits one copy of each parental nucleus with mirror image symmetry.

What is the mechanism of nuclear migration?

Centrin is a calcium binding protein associated with basal bodies and centrosomes of eukaryotic cells (Salisbury 1995). In algal cells, centrin is a major component of the nuclear basal body connector, a fibrous network that physically links the basal bodies to the nucleus.

Centrin was demonstrated in *Giardia* (Correa *et al*. 2004; Sagolla *et al*. 2006). The reports have demonstrated that centrin was localized in basal bodies, in posterior-lateral rods, in the funis and in the median body. Sagolla *et al*. (2006) suggested that centrin could represent a link between flagellar segregation and nuclear migration.

An approach using plasma membrane extraction allowed the observation of the two nuclei still attached in their original positions (Benchimol 2005). Both nuclei are anchored by fibrillar connections to basal bodies of the anterior flagella and to the descending posterior-lateral and ventral flagella, at the right and left nuclei, respectively, in cells attached by its ventral disc. This attachment occurs by proteinaceous links, which are labeled by anti-actin and anti-centrin but not by anti-dynein or anti-tubulin anti-

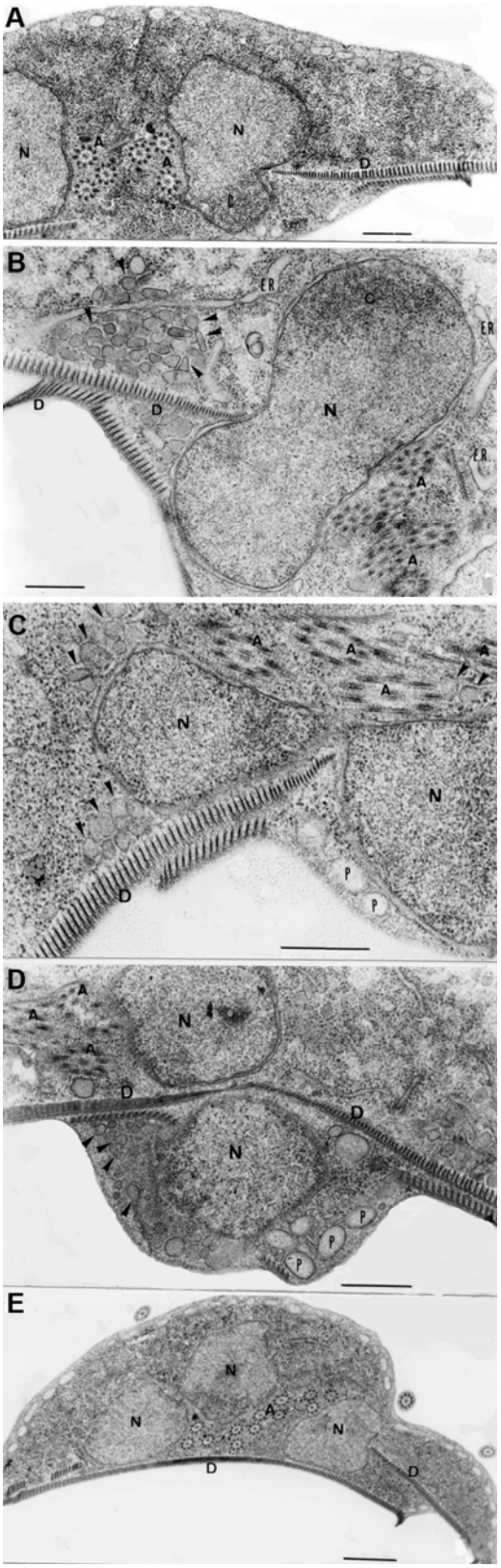

Fig. 21 Participation of the ventral disc (D) in the process of karyokinesis. Note that a disc fragment contacts one nucleus (N), pressing it until complete separation (**A-E**). The nucleus in division first elongates while the disc presses it. Smooth vesicles are also observed and are known as internal vesicles (arrowheads). A, axonemes; ER, endoplasmic reticulum; P, peripheral vesicles. From Benchimol (2004a) *Biology of the Cell* **96**, 291-301, with kind permission from Publisher's name.

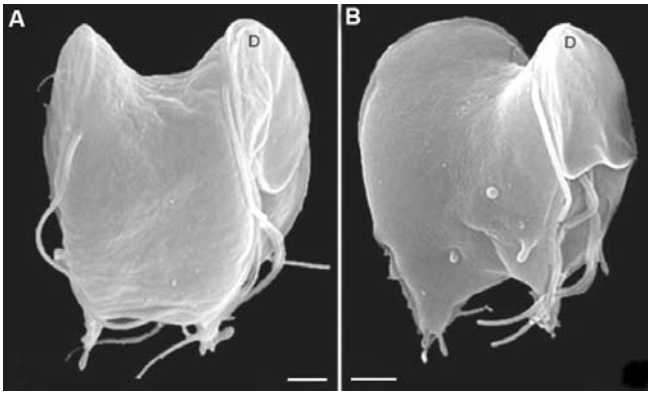

Fig. 22 Scanning electron microscopy of the dorsal-dorsal mode of division in *Giardia*. The daughter cell is formed on the dorsal region of the parental cell and is gradually separated. In this type of division the cell is weakly adherent and easily detached. In this mode of division *Giardia* replicate with mirror-image symmetry. D, ventral disc. Bar = 1 μm. From Benchimol (2004c) *Protist* **155**, 33-44, with kind permission from Elsevier.

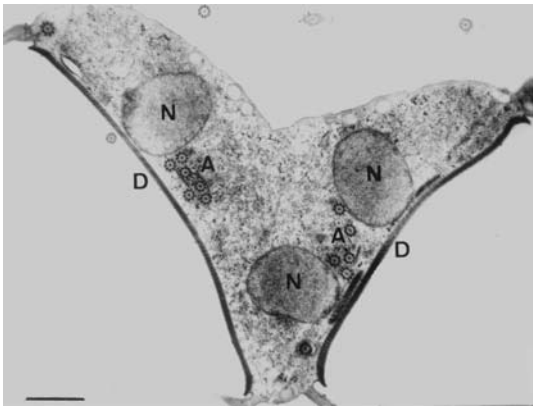

Fig. 23 Transmission electron microscopy of the dorsal-dorsal mode of division. Note that the discs (D) are in opposite positions. Bar = 1μm. From Benchimol (2004c) *Protist* **155**, 33-44, with kind permission from Elsevier.

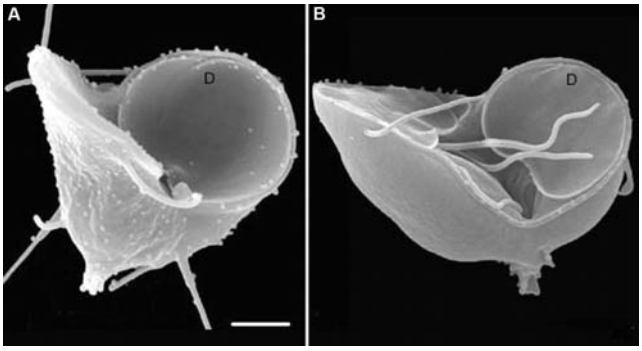

Fig. 24 Scanning electron microscopy of the ventral-ventral mode de division. Note that the discs (D) are facing each other. Bar = 2 μm. From Benchimol (2004c) *Protist* **155**, 33-44, with kind permission from Elsevier.

bodies (Benchimol 2005). Interestingly, the nuclei exhibited a pendular movement when living cells were treated with cytochalasin, although they were still connected by their anterior region. All these analyses indicated that the nuclei have a defined position and fibrils perform an anchoring system. Thus, it is possible that nuclei migration during mitosis occurs with fidelity due the links between the nuclei and basal bodies of the flagella axonemes (Benchimol 2005).

Table 2 Proposals for *Giardia lamblia* mitosis phases.

Prophase	Metaphase	Anaphase	Telophase	Cytokinesis
Chromatin condensation [9-10]	Nuclei become stacked along the dorsal-ventral axis [10]	Lateral chromosome segregation in two stages [10] Anaphase A: chromosomes are segregated to spindle poles along the left-right axis of the cell [10]	Nuclei remain physically and genetically distinct from one another [10]	DNA decondenses [10]
Nucleation of spindle microtubules [10]	Microtubules organized into bipolar spindles [9-10]	(3) Anaphase B spindle elongation along the left-right axis of the cell [10]	Daughter cells inheriting one copy of both nuclei [10]	Multiple modes of cytokinesis [8] Cytokinesis occurs along the longitudinal axis – perpendicular to the spindle [10]
Each chromosome contained a single centromeric locus [10]	Two independent bipolar spindles, stacked in the dorsal-ventral plane [10]	Centromeres clustered at the spindle poles [10]	Centromeres remain clustered [10]	Centromeres foci throughout the nucleus [10]
Centrin foci duplication [10]	Centrin foci are at the four spindle poles bipolar spindles [10]	Centrin foci remained at the spindle poles throughout anaphase A and anaphase B [10]	The two centrin foci moved from near the cell periphery to between each pair of nuclei as seen in interphase[10]	Centrin stains two foci between the nuclei [10]
Repositioning of the nuclei; nuclei migrate to the cell midline [10]	The anterior flagella is wrapped around the dorsal nucleus [10]	At completion of anaphase, nuclei of different parental origin reside on opposite sides [10]	A linear microtubule structure replaces the bipolar spindles [10]	A cleavage furrow forms at the anterior end, creating a heart-shaped cell [10]
Flagella rearrangement [9, 10]	Gradual migration and transformation of parent flagella[9, 10]	Gradual migration and transformation of parent flagella[9, 10]	Gradual migration and transformation of parent flagella [9, 10]	The basal body/flagellar maturation is spread over three successive cell cycles[9]
One nucleus is placed on top of the other along the dorsal-ventral axis [10]	No observation of metaphase alignment of centromeres along a metaphase plate [10]	Each daughter cell inherits two nuclei each derived from one left and one right nucleus of the mother cell [10]	The single microtubule bundle has unfocused ends [10]	The furrow progressed from anterior to posterior, in the longitudinal plane. Cell is divided into left and right halves [10]
Nuclei remain separate [1-10]	The opposing poles of each spindle were oriented along the left-right axis of the cell [3, 10]	Nuclear envelope elongates with the spindle in anaphase B [10]	The microtubule bundle extends between the nuclei on each side of the cell [10]	Daughter cells inherit one copy of each parent nucleus [7, 10]
Centrin foci at the sites of microtubule nucleation [10]	Persistence of the nuclear envelope throughout mitosis [1- 2, 7-10]	Persistence of the nuclear envelope throughout mitosis [1- 2, 7- 10]		Cells in mirror-image symmetry [1, 2, 4, 5, 7, 8, 10]
Apparently nuclei do not exchange genetic information [1-10]	Mitosis is semi-open [9-10]			Regeneration of all cytoskeletal structures [no identified yet]
Karyokinesis occurs with the disc aid [6,7] along the left-right axis [10]				Nuclei adjacent to one another
Appearance of two daughter nuclei, one from each parental nucleus [4-5, 7, 10]				Each daughter cell inherits one parental and one new formed nucleus [7-10]
Lateral movement of the two parent kinetosomal tetrads [3]				
Presence of two extranuclear central mitotic spindles [10]				
Disc participation on karyokinesis [3, 4]				
Spindle attachment to the chromosomes at the kinetochore [10]				
During nuclear migration the spindle microtubules continued to elongate [10]				
Spindle microtubules enter the nuclei through polar openings in the nuclear envelopes [3, 10]				

References: (1) Filice 1952; (2) Cèrva and Nohýnková 1992; (3) Nohýnková *et al*. (2000); (4) Ghosh *et al*. (2001); (5) Yu *et al*. (2002); (6) Solari *et al*. (2003); (7) Benchimol (2004a); (8) Benchimol (2004b); (9) Nohýnková *et al*. (2006); (10) Sagolla *et al*. (2006).

Nuclei inheritance

It was demonstrated that the *Giardia* nucleus is connected to the anterior flagella, to the posterior-lateral and ventral flagella, by the right and left sides, respectively (**Fig. 14**; Benchimol 2004b). The consequence of nuclei being connected to these flagella axonemes is that these structures can migrate together during mitosis. These links would explain the behavior of parental nuclei, which can be maintained together during the dividing process, whereas the daughter nuclei are distributed to the new cell. Thus, this finding would confirm that the two nuclei are partitioned equationally at cytokinesis as published before by Ghosh *et al*. (2001) and Yu *et al*. (2002). Sagolla *et al*. (2006) pro-

posed using fluorescence in situ hybridization (FISH) to an episomal plasmid published that during mitosis the *Giardia* nuclei remain separate and are inherited with mirror image symmetry.

Flagella inheritance

Nohýnková *et al*. (2006) examined the cell division of *G. intestinalis* with the aid of light and electron microscopy and immunofluorescence methods and presented observations on the reorganization of the flagellar apparatus in this dividing protist. They proposed a flagellar maturation process during which the flagella migrate, assume different positions and transform to different flagellar types in progeny until their maturation is completed. In addition, the authors stated that for each newly assembled flagellum it takes three cell cycles to become mature. The mature caudal flagellum of *Giardia* possesses a privileged basal body at which the microtubules of the adhesive disk nucleate. In contrast to the generally accepted assumption that each of the two diplomonad mastigonts develops separately, this group found that they are developmentally linked, exchanging their cytoskeletal components at an early phase of mitosis. This group also stated that during division each daughter *Giardia* receives four flagella from the parent cell that are supplemented to the full eight-flagellate set by new flagella which arise *de novo*. Contrary to other groups, Nohýnková and colleagues (2006) claimed that before mastigont separation, a half of each mastigont component migrates to the opposite cell side, whereas the other half retains its interphase position. Consequently, the parent set inherited by progeny is composed of a half of each parent mastigont. Sagolla *et al*. (2006) agreed with these findings and added that the dramatic rearrangement of the flagella coincided with nuclear migration in prophase.

BEHAVIOR OF CYTOSKELETAL STRUCTURES DURING MITOSIS

The median body in mitotic cells

The median body is a microtubular structure located dorsally in the cytoplasm behind the disc with unknown function. It has been demonstrate that median bodies (MB) persist throughout cell cycle phases, mitosis included (**Fig. 8**) (Piva and Benchimol 2004; Sagolla *et al*. 2006). These data are contradictory to early studies (Soloviev 1963; Filice 1952; Cerva and Nohýnková 1992). Soloviev (1963) reported the absence of the median bodies in the late phases of division and in young daughter individuals, whereas Filice (1952) stated the absence of the MB in freshly excysted trophozoites. Cerva and Nohýnková (1992) reported that the median bodies disappear in early phases of *Giardia* mitosis (**Table 2**), before karyokinesis.

Meiosis in *Giardia*?

The recent identification of putative meiotic genes in *Giardia* (Ramesh *et al*. 2005) has raised the possibility that rare nuclear fusions or meiotic events provide a mechanism for chromosomal recombination and reduction of such heterozygosity. This group surveyed the ongoing *G. lamblia* genome project data and have identified, verified, and analyzed a core set of putative meiotic genes-including five meiosis-specific genes-that are widely present among sexual eukaryotes. Thus, the authors (Ramesh *et al*. 2005) raised the possibility that *Giardia* is capable of meiosis, and thus sexual reproduction. In addition, they proposed that the evolution of meiosis occurred early in eukaryotic evolution, and the conserved meiotic machinery comprises a large set of genes, including those involved in meiotic recombination.

OPEN QUESTIONS

Although molecular data and the genome project have provided advances in *Giardia* knowledgement such as the discovery of nuclear genes of putative mitochondrial ancestry in *Giardia* and the discovery of components of protein machinery responsible for iron-sulfur cluster assembly (Roger *et al*. 1998; Morrison *et al*. 2001; Tachezy *et al*. 2001; Arisue *et al*. 2002; Tovar *et al*. 2003) allowing a suggestion that the eukaryotic amitochondrial state is not a primitive condition but is rather the result of reductive evolution, there are still several questions to be answered concerning *Giardia* division. Among them are: (1) how the two nuclei are divided; (2) how the eight flagella are duplicated; (3) what the spindle is like: how many microtubules, how the spindle microtubules interact with the chromosomes; (4) how the chromosomes are partitioned; (5) how the nuclear envelope participates in the mitotic process; (6) if the division process is already started before or after the encystation; (7) if there is one or multiple division planes; (8) how the genetic heterozygosity is maintained; (9) if *Giardia* presents meiosis; (10) how a new disc is formed; (11) what the behavior of the cytoskeletal structures during mitosis: disc, median body, funis; (12) when and how the cytoskeletal structures are duplicated; (13) what about the morphological evidence of *Giardia* kinetochores; (14) evidence of kinetochore-microtubule associations; (15) how many microtubules for each kinetochore; (16) what role the flagellar basal bodies play in the spindle organization; (17) what the nuclear migration mechanism is; (18) which basal body the MTOC is; (19) what about the ultrastructural evidence of chromosomes; (20) and evidence of the chromosomes congress to the spindle midzone during metaphase.

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