How Giardia Swim and Divide

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To determine how binuclear giardia swim, we used video microscopy to observe trophozoites of *Giardia intestinalis*, which were labeled with an amino-specific Alexa Fluor dye that highlighted the flagella and adherence disc. Giardia swim forward by means of the synchronous beating of anterior, posterolateral, and ventral flagella in the plane of the ventral disc, while caudal flagella swim in a plane perpendicular to the disc. Giardia turned in the plane of the disc by means of a rudder-like motion of its tail, which was constant rather than beating. To determine how giardia divide, we used three-dimensional confocal microscopy, the same surface label, nuclear stains, and antitubulin antibodies. Giardia divided with mirror-image symmetry in the plane of the adherence disc, so that the right nucleus of the mother became the left nucleus of the daughter. Pairs of nuclei were tethered together by microtubules which surrounded nuclei and prevented mother or daughter giardia from receiving two copies of the same nucleus. New adherence discs formed upon a spiral backbone of microtubules, which had a clockwise rotation when viewed from the ventral surface. These dynamic observations of the parasite begin to reveal how giardia swim and divide.

*G. intestinalis* (also known as *Giardia lamblia*), which was likely first visualized by von Leeuwenhoek, is a protist that causes intestinal malabsorption and diarrhea (3, 21). Although giardia cause a unattractive disease, they are among the most beautiful organisms, as shown by scanning and transmission microscopy (4, 7, 10). Trophozoites of *G. intestinalis* have two similar-appearing nuclei, which are both transcriptionally active (13). The giardia nuclei are bilaterally symmetric, as are four other microtubule-associated structures: the ventral adherence disc, four pairs of flagella, the median body, and the funis (2, 7, 10). The ventral disc, by which giardia adhere to the surface of intestinal epithelial cells, is composed of α- and β-tubulin and at least three different unique cytoskeletal proteins called giardins (2, 12, 17, 18, 20). Giardia have four pairs of flagella (anterior, posterolateral, ventral, and caudal), which are composed of microtubules in a 9-plus-2 arrangement (7, 10). All four pairs of flagella originate from basal bodies, composed of microtubule triplets, which are located between the two nuclei and are dorsal to the adherence disc. The funis is a set of single microtubules, which run parallel to the caudal flagella from the disc to the tip of the tail (2). The median body, which is a bundle of microtubules bound by a unique protein called the median body protein, is perpendicular to the funis and caudal to the adherence disc (15).

Holberton (10) used phase microscopy to show that the ventral flagella of adherent giardia were constantly beating in a synchronized manner in the plane of the adherence disc. Electron micrographs of adhering giardia to mouse intestines suggested that parasites were drawing the intestinal villi up to the adherence disc. Holberton proposed that the motion of the ventral flagella creates a vacuum under the disc that sucks the intestinal epithelium to the giardia (11). It is likely that lectins on the surface of giardia also bind sugars on the surface of intestinal epithelial cells (5). Because of the limits of phase microscopy, Holberton was unable to determine the motion of the ventral flagella when giardia swim or divide and was unable to determine the motion of the anterior, posterolateral, and caudal flagella. Here we used video microscopy and amino-specific Alexa Fluor dyes, which were recently used to demonstrate the motion of bacterial flagella (23), to determine the motion of each pair of flagella of adherent and swimming giardia.

As giardia are motile and nonadherent when they divide, little is known about how the organisms replicate themselves. For example, early investigators thought that mother and daughter giardia divide along a sagittal plane, so that the mother got two identical copies of one nucleus while the daughter got two identical copies of the other nucleus (6). Others suggested that the daughter giardia slid off the back of the mother, so that the left nuclei of mother and daughter giardia were the same (when viewed from the dorsal surface) and that the right nuclei were the same (13). In contrast, our results here suggest that giardia divide with mirror-image symmetry in the plane of the adherence disc, so that the left nucleus of the mother becomes the right nucleus of the daughter. Further, we used antitubulin antibodies to identify perinuclear tethers, which bind pairs of nuclei together during cell division.

**MATERIALS AND METHODS**

Labeling surface of giardia. The WB strain of *G. intestinalis* was grown axenically at 37°C in TY1-S-33 medium supplemented with bile salts (14). Dividing parasites, which are motile and nonadherent, were collected by removing the supernatant of unchilled cultures. Nondividing giardia, which are adherent to the plastic culture flask in logarithmic-phase cultures, were collected by adding ice-cold phosphate-buffered saline (PBS), pH 7, to the remaining parasites. For studies of giardia cysts, organisms were incubated in encystation medium for 24 h, as described earlier in reference 9. Cysts were recognized by their characteristic ovalness, the absence of flagella, and the presence of four nuclei. Giardia were washed in PBS; placed in 100 μl of PBS, pH 8.5, to maximize the number of deproteinated amino groups on their surface; and incubated for 1 h at 37°C.

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with 0.25 mg of Alexa Fluor 488 or Alexa Fluor 584 (Molecular Probes, Eugene, Ore.). Alexa Fluor dyes are carboxylic acid succinimidyl esters, which react with deproteinized amino groups (23). Alexa Fluor 488, which fluoresces like fluorescein, was predominantly used for confocal microscopy studies. Alexa Fluor 584, which fluoresces like Texas red, was predominantly used for video microscopy, because it tended to bleach less. After labeling, giardia were washed in PBS four times and were then returned to complete culture medium.

**Video microscopy.** Surface-labeled giardia were observed with a Nikon Eclipse TE 300 inverted-fluorescence microscope, to which was attached a Hamamatsu digital video camera, a Lambda 10-2 controller (Sutter), and a MetaMorph (Universal Imaging) shutter and image analysis system. Digital recordings were made of swimming giardia using 60× or 100× objectives and the streaming mode, which captured ~20 images per s. The zoom feature was used to focus and enlarge individual giardia, while individual frames were examined to determine the motion of each pair of flagella. Dozens of hours of observations and recordings were distilled into the video micrographs shown (see Fig. 2 and 3).

**Labeling cytoskeleton and nuclei of giardia.** WB giardia, some of which were labeled on their surface with Alexa Fluor 488, were fixed in 2% formaldehyde at 4°C for 10 min, washed in PBS, and permeabilized with 0.1% Triton X-100 for 1 to 2 min. To highlight different structures in dividing giardia, parasites were stained with three different antibodies to α-tubulin. Some giardia were incubated for 1 h at 17°C with a mouse monoclonal antibody to bovine α-tubulin (Molecular Probes), which was diluted 1:100 in PBS with 2 mg of bovine serum albumin/ml. The giardia were washed four times in PBS and were then incubated for 1 h at room temperature with a rhodamine-conjugated goat anti-mouse antibody. Alternatively, some giardia were incubated with a polyclonal rabbit antibody to α-tubulin (Sigma), diluted 1:100, which was detected with a Texas red-conjugated goat anti-rabbit antibody (20). Finally, some giardia were incubated with a mouse monoclonal anti-α-tubulin antibody, diluted 1:100, which was also detected with a rhodamine-conjugated goat anti-rabbit antibody. In addition, some giardia were incubated with 1 μg of propidium iodide or Sytox green per ml to label the nuclei.

**Three-dimensional confocal laser scanning microscopy.** Immunolabeled giardia were placed in mounting media, coveredslipped, and examined using a Leica TCS NT confocal laser scanning microscope (Leica Inc., Exton, Pa.). A bandpass (530 + 30 nm) filter was used to select light emitted from the nucleus-specific probe (Sytox green), and a long-pass 590-nm filter was used to detect the rhodamine- and Texas red-conjugated antibodies under multiple-channel fluorescent mode. Serial sections were collected from the apical surfaces of giardia with step increments of 0.5 to 1 μm in the z axis. Three-dimensional reconstructions which simultaneously showed features on the inside and outside of giardia were created using VoxelView (Vital Images, Fairfield, Iowa). Projections were electronically repositioned to view the opposite side of these organisms.

**Scanning electron microscopy.** Giardia were fixed in 2% glutaraldehyde in PBS, postfixed in 1% osmium tetroxide, dehydrated in a graded ethanol series to absolute followed by hexamethyldisilazane, and vacuum desiccated. Dry specimens were distilled into the video micrographs shown (see Fig. 2 and 3).

**Anatomy of giardia labeled on surface with amino-specific Alexa Fluor dyes.** Alexa Fluor dyes, which have recently been used to study the motion of bacterial flagella during running and tumbling (23), highlighted four pairs of giardia flagella with no apparent effect on viability or cell division (Fig. 1A).

The relationship between surface labels and underlying cytoskeletal structures was shown by three-dimensional confocal microscopy (Fig. 1B). Anterior flagella cross the midline, exit the cytoplasm at the lateral borders of the adherence disc, and extend 8 μm. Ventral flagella, which look thicker than the other flagella, exit the cytoplasm at the posterior border of the disc and extend 12 μm. Posterolateral flagella, which are contained within the caudal cytoplasm for about half their length, exit at the side of the tail and extend 8 μm. Caudal flagella, which are for the most part contained within the cytoplasm, exit at the end of the tail and extend just 4 μm.

**Anterior, posterolateral, and ventral flagella of giardia beat with a similar frequency in the plane of the adherence disc.** Giardia prefer to adhere to a surface rather than swim freely in the medium. For the most part, anterior, posterolateral, and caudal flagella of adherent giardia were still (Fig. 2A and 2B). In contrast, the ventral flagella of adherent giardia were constantly beating together in the plane of the adherence disc. The waveform of the flagellar beating was bilaterally symmetric and advanced from the caudal edge of the adherence disc to the tips of the flagella. The waveform had an amplitude of 1.7 μm, a wavelength of 5.8 μm, and a frequency of 5 to 10 beats/s, depending upon the temperature of the medium and the freshness of the parasite preparation. When giardia swam in the direction of the adherence disc, ventral flagella moved with the same bilaterally symmetric, pseudosinusoidal waveform as that of adherent giardia (Fig. 2C). Posterolateral flagella beat in the plane of the adherence disc with a frequency and wavelength similar to those of ventral flagella, but the amplitude was smaller (1.1 μm) (Fig. 2B). The anterior flagella beat in nearly the same plane and with the same frequency as ventral and posterior flagella, but the bending of the anterior flagella was asymmetric (Fig. 2C). Movies of swimming giardia are available for viewing at http://www.hsp.harvard.edu/physiology/projects/bilavi/.

**Two motions (beating and turning) of tails of swimming giardia.** For the most part, the tail of adherent giardia was still, with the distal tips of the caudal flagella projecting like two short antennae (Fig. 1A and B). In contrast, the tail of swimming giardia beat in the plane perpendicular to the adherence...
disc with the same frequency as the beating of ventral, anterior, and posterolateral flagella. When swimming giardia were viewed from the dorsal or ventral surfaces, their tails moved in and out of the plane of focus (Fig. 2C). In contrast, when swimming or dividing giardia were seen in profile, their tails moved up and down (Fig. 2D and E, respectively). Giardia swam straight in the plane of the adherence disc (Fig. 2C) and/or rotated clockwise or counterclockwise along their long
axis (Fig. 3A and B). Alternatively, giardia turned in the plane of the adherence disc by curving their tails like the rudder of a boat (Fig. 2F). When giardia turned, the caudal flagella, which were not beating, were parallel to each other. Giardia maintained their curved shape for a number of seconds, so that the parasites turned in multiple circles.

**Giardia replicate with mirror-image symmetry in plane of adherence disc.** Dividing giardia had a mirror-image symmetry in the plane of the disc, so that it was impossible to distinguish the mother from the daughter. Mirror-image symmetry was present whether discs faced toward, away, or in the same plane as the others (Fig. 4A to C). Giardia also showed mirror-image symmetry when the cytoskeleton rather than the surface was visualized with antibodies to acetylated tubulin (Fig. 4D). A prediction of the mirror-image symmetry hypothesis is that the left nucleus of the mother should become the right nucleus of the daughter giardia. This was shown by introducing a foreign episome into a single nucleus of transfected giardia and localizing the episome using FISH (16, 19). A quadrinucleate, mitotic organism had episomal plasmids in the right nucleus of one pair of nuclei and the left nucleus of the other pair of nuclei (Fig. 5A). The episome-associated nucleus could be either left or right when viewed from the dorsal surface of giardia, which adhered to poly-Lys-coated slides prior to fixation (Fig. 5B). The ~50:50 mixture of left- and right-labeled nuclei was maintained after transfected WB was recloned on soft agar (8). A summary of the FISH results is shown in Fig. 5C.

**Pairs of nuclei are tethered together when giardia replicate.** Nuclei of nondividing giardia were surrounded by perinuclear tethers, which were composed of microtubules that were highlighted by a polyclonal rabbit anti-α-tubulin antibody (Fig. 6A to C). Microtubules within the perinuclear tethers did not appear to be acetylated, because they were poorly visualized with antibodies to acetylated tubulin (Fig. 4D and 7B and C) (20). During cell division, both pairs of nuclei were surrounded by perinuclear tethers of microtubules (Fig. 6D). These perinuclear tethers also surrounded the nuclei of giardia cysts (Fig. 6E). Three-dimensional reconstructions of confocal images suggested that perinuclear tethers were developed from a set of microtubules, which ran along the central axis of giardia (Fig. 6F).

**Spiral symmetry of new adherence discs.** The ventral adherence disc of nondividing giardia contained a clockwise spiral marking, which was visible with scanning electron microscopy (Fig. 7A). This spiral is made up in part by acetylated tubulin, as shown by confocal microscopy of giardia, which were also labeled on their surface with Alexa Fluor 488 (Fig. 7B) or on their nuclei with Sytox green (Fig. 7C). During cell division, the adherence disc of the mother disassembled, so that dividing giardia often appeared as ovals with eight pairs of projecting flagella (not shown). New adherence discs developed from a spiral backbone of microtubules, which ran clockwise when viewed from the ventral surface of the parasites. (Fig. 7D and E). Three-dimensional reconstructions of confocal images suggested that the spirals developed from a set of microtubules which ran along the central axis of giardia (Fig. 7F). The adherence discs and flagella were all well formed before mother and daughter giardia separated from each other.
DISCUSSION

A more complete description of motion of flagella of adherent and swimming giardia. The wavelike beating of the anterior, posterolateral, and caudal flagella was dramatically revealed when the surfaces of giardia were labeled with the Alexa Fluor dyes (23). As previously described (10), the ventral flagella had a pseudosinusoidal beat, which was symmetric in the plane of the adherence disc. Consistent with Holberton’s vacuum model of giardia adherence (10, 11), fluorescent beads were sucked from the side of the parasite up under the adherence disc and were shot to its caudal end. In addition, giardia

FIG. 5. FISH with a probe to a foreign plasmid in transfected giardia. (A) Fluorescence micrograph of a quadrinucleate, dividing giardia which has plasmids (yellow) on the right side of one pair of nuclei and on the left side of the other pair. (B) Confocal micrographs of transfected and cloned giardia, which show that the episomal plasmids (yellow) are present in just one nucleus, which may be left and right. Bars, 2 μm. (C) Cartoon shows that giardia divide with mirror-image symmetry, so that episomal plasmids in the right nucleus of the mother are present in the left nucleus of the daughter.

FIG. 6. Three-dimensional confocal micrographs highlighting microtubules which form perinuclear tethers. (A to C) Micrographs of a giardia which was stained with polyclonal antibodies to α-tubulin (red in panels A and C) and Sytox green (green in panel B and yellow in panel C) show tethers of microtubules (arrows) that surround both nuclei. A dividing giardia (D) and an encysted giardia (E) stained with the same antitubulin antibodies have prominent perinuclear tethers (arrows). Note that the wall of the cyst is nonspecifically stained with the antibodies. Bars, 2 μm. (F) Cartoon of perinuclear tethers of microtubules which connect to microtubules in the central axis of the parasite.
adhered to but slid along the surface of silenized slides. Three unexpected findings follow: first, all four pairs of flagella of swimming giardia appeared to beat at about the same rate, even though the shape and/or the plane of the beat was different for each. How the beating is “centrally controlled” remains to be determined. Second, the tails of giardia moved up and down in a plane perpendicular to the plane of the adherence disc. It is likely that this movement was caused by the caudal flagella beating synchronously together, because caudal flagella are present throughout the entire length of the tail and because the beating of the tail was at the same rate as the beating of the other three pairs of flagella. Third, giardia turned by using their tail as a rudder rather than by adjusting the stroke of the flagella. This is in contrast to Chlamydomonas, which turns by changing the symmetry of the beat of its two flagella, or Paramecia, which turns by changing the stroke of its many cilia, or bacteria, which turn by tumbling (1, 23). Because turning giardia maintain their curved shape for a long time, it is likely that the median body and/or funis microtubules are responsible for turning rather than the caudal flagella.

Mirror-image hypothesis for cell division by giardia. How giardia replicate their nuclei and distribute them to mother and daughter giardia has been debated for a long time (6, 13). The surface and cytoskeletal labels used here strongly suggest that giardia are replicating with mirror-image symmetry in the plane of the adherence disc. The mirror-image hypothesis was confirmed by FISH, which showed that an episomal plasmid was present in the right nucleus of a transfected mother giardia and the left nucleus of the daughter, while a set of cloned giardia contained a 50:50 mixture of parasites with epismes in the left and right nuclei. The previous models of cytokinesis by giardia are incorrect because they predict that both nuclei or no nuclei will contain the epismes (6) or predict that each giardia clone will have all foreign epismes in the left nucleus or the right nucleus but not in a 50:50 mixture of left and right nuclei (13).

Two new cytoskeletal structures which are likely involved in sorting nuclei of dividing giardia and building the disc. One copy of each nucleus is appropriately distributed to mother and daughter giardia, because at least one pair of nuclei is bound at all times by the perinuclear tethers, which are composed of nonacetylated microtubules. In the absence of these tethering microtubules, 50% of the mother and daughter giardia would be homonucleate, which might dramatically reduce the genetic diversity of this asexual parasite (22). The adherence discs, which are composed of microtubules and ribbons of giardins (2, 12, 17, 18), appear to develop on a spiral backbone of acetylated microtubules. Although giardia generally divide...
with mirror-image symmetry in the plane of the adherence disc, the spirals always turn clockwise when discs are viewed from the ventral surface. While perinuclear tethers and spirals appear to originate from a central axis of microtubules, which joins the funis at its caudal end, the relationship of tethers and spirals to each other was difficult to determine. It is likely that future studies will reveal specific microtubule-associated proteins which assist in the assembly of tethers and spirals in the same way that dineins, giardins, and median body proteins direct microtubules in flagella, the adherence disc, and the median body, respectively (12, 15, 17, 18).

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