

Subpellicular Microtubules in Apicomplexa and Trypanosomatids

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Abstract The cytoskeleton plays a fundamental role in various processes such as the establishment of cell shape, cell locomotion, and the intracellular motility of various structures found in eukaryotic cells. Microtubules are among the most conspicuous structures in the cytoskeleton. They can be found free in the cytoplasm, forming the mitotic spindle or assembled in various structures. A special type of microtubule arrangement is found in some protozoa, whereby they are organized as a single layer located immediately below the plasma membrane, constituting what is generally referred to as subpellicular microtubules. This special array of microtubules is found in members of the Kinetoplastida family and in the Apicomplexa phylum. Here, we review basic aspects of subpellicular microtubules, emphasizing their visualization as a whole network, their structural organization, their heterogeneity as analyzed using an immunocytochemical approach, some of their biochemical properties, and their sensitivity to drugs, as well as their functional role.

1 Introduction

Microtubules can be defined as a macromolecular complex in which the major proteins (tubulins) associate with each other, building a polymeric structure that interacts with minor proteins; these, in turn, control the stability and the dynamic assembly–disassembly process of microtubules, modulating their functional properties (Fig. 1) (Reviewed in Li and Gundersen 2008). The length of the polymer, which in some cases is essential for its function, is regulated by the addition and/or loss of tubulin subunits at the plus and minus ends of the microtubule, respectively, and breakage of the polymers by severing enzymes such as katanin, spastin, and fidgetin (Casanova et al. 2009).

In most eukaryotic cells, the microtubules appear as individualized structures. However, they may associate with each other and to other cell structures to form more complex assemblies. Cilia and flagella, found in many cell types in organisms ranging from protists to mammals, are two very representative and well-known examples of such structures. However, protists' microtubules can assemble in a wider variety of patterns and form unique and complex structures. Examples include the pelta–axostylar system found in the members of the Trichomonadidae family (see chapter “The Mastigont System in Trichomonads” by Benchimol), the adhesive disk of *Giardia*, and the subpellicular microtubules found in Apicomplexa and Kinetoplastida.

This chapter will deal with subpellicular microtubules. These are found in two groups of protists of high relevance, as they include the agents of some very important human and veterinary diseases: Kinetoplastida and Apicomplexa.

The Apicomplexa phylum comprises a large and heterogeneous group of species that are distributed worldwide and in a variety of environments. Many of them are agents of important diseases affecting humans, as is the case for the agents of malaria (*Plasmodium* genus) and toxoplasmosis (*Toxoplasma gondii*). Others cause disease in animals of economic interest (*Eimeria* in chickens and cattle, *Theileria* in

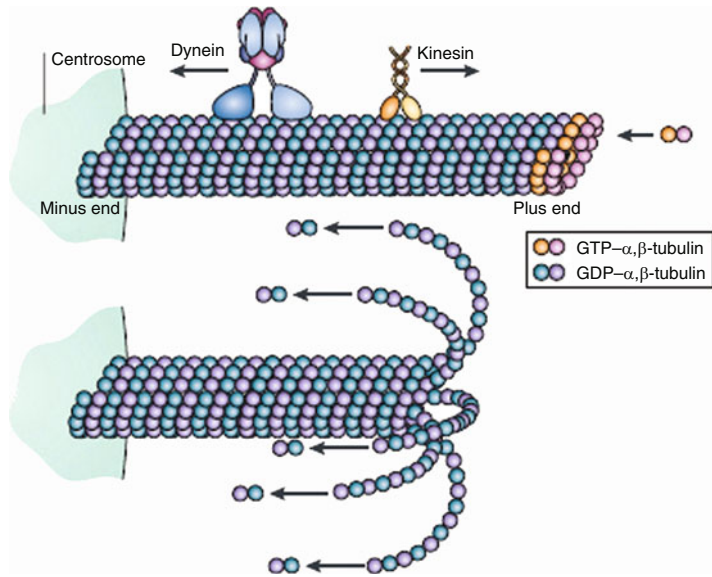


Fig. 1 Schematic representation of the main features of microtubules: heterodimers of $\alpha\beta$ -tubulin are assembled from the centrosome, or MTOC, with the minus ends toward it and the plus ends to the cell periphery. Motor proteins may use microtubules as tracks to transport molecules or organelles in specific directions: toward the centrosome, in the case of dyneins, or to the cell periphery, for kinesins. The microtubule grows as cytoplasmic dimmers of $\alpha\beta$ -tubulin associated with GTP are added to the plus ends. After assembly, GTP is hydrolyzed to GDP weakening the microtubule and favoring its quick depolymerization. This alternation between growth (*upper panel*) and shrinkage (*bottom*) is known as dynamic instability (Adapted from Li and Gundersen 2008 with permission)

cattle). The Apicomplexa phylum also includes *Cryptosporidium*, a parasite that infects animals and is also an opportunistic parasite of humans, and nonpathogenic protozoa such as *Gregarina*. Among the pathogenic species, some present a life cycle that involves a single animal species. Others, as is the case of *T. gondii*, have a more complex life cycle involving certain animal species in which only the asexual cycle takes place and other species in which the life cycle is complete. Still others, as is the case of *Plasmodium*, have an even more complex life cycle involving vertebrate and invertebrate (insect) hosts.

The Kinetoplastidae order comprises a large number of species, some of which are the causative agents of highly prevalent diseases in humans, such as leishmaniasis, Chagas' disease and sleeping sickness. Species of the genus *Phytomonas* also affect plants of economic interest, such as coconut, oil palm, and cassava.

2 Subpellicular Microtubules

The basic scaffold that determines the cell shape in several protists, including Trypanosomatids and Apicomplexans, is composed of parallel rows of microtubules that run under the pellicle – the subpellicular microtubules. This and the

fact that in both cases, tubulin dimmers are the main constituents are two of the few characteristics shared by subpellicular microtubules of Trypanosomatids and Apicomplexa.

2.1 In Trypanosomatids

Trypanosomatids belong to the order Kinetoplastida – that comprises the suborders Bodonina and Trypanosomatina. The first includes both parasitic and free-living genera, such as *Cryptobia* and *Bodo*, respectively. Although *Cryptobia* species are parasites of salmonid fishes with considerable impact on fish-farming, this group has not received much attention so far. However, Trypanosomatids, especially the trypanosomes, were already notorious in the early history of electron microscopy. In 1950, Hertha Meyer started to study *Trypanosoma cruzi* with an electron microscope in the laboratory of Keith Porter at the Rockefeller Institute in New York (Meyer and Porter 1954; Review in De Souza 2008). At that time, it was not possible to obtain thin sections, although many groups were trying to adapt a conventional microtome to cut thinner sections. The first attempts used tissue cultures infected with *T. cruzi* or culture forms of the protozoan. The samples were fixed with osmium tetroxide and washed several times, and then one drop of the cell suspension was placed on a copper grid previously coated with Parlodion and allowed to dry in dust-protected dishes. The first results obtained were rather disappointing. In spite of the small size of the flagellates, much smaller than a tissue cell, nothing could be seen of their inner structure. Only at the periphery was the cell thin enough to be traversed by the electron beam, revealing fine striations in a parallel array in the cytoplasm (Meyer and Porter 1954). Trypsinization or prolonged fixation with osmium tetroxide destroyed this fine striation in the periphery of the cell as well as the fiber bundle of the flagellum. The above-mentioned striations correspond to what are now known as subpellicular microtubules, the major component of the cytoskeleton of trypanosomatids. Subsequently, with the methodological improvements in the fixation of biological specimens for electron microscopy, these microtubules were better visualized, especially after the introduction of glutaraldehyde as a fixative.

Transmission electron microscopy of thin sections showed that, in all species, the microtubules have a diameter of around 24 nm and they keep a fixed distance of about 44 nm between each other and about 15 nm with the plasma membrane (Fig. 2). A detailed study of *T. cruzi* showed that the number of microtubules varied according to the regions of the protozoan's body. In trypomastigote forms, the maximum number of microtubules found was 120 in the region where the Golgi complex is located (Meyer and De Souza 1976). At the cell extremities, as few as 40 microtubules were counted. In the proliferative amastigote forms, the highest number of microtubules found was 222, observed in dividing cells. This observation indicates that there is an increase in the number of microtubules during the cell division process, and suggests that the assemblage of microtubules is organized and controlled in such a way that, as the volume of the cell increases, new microtubules



Fig. 2 Cross-section of subpellicular microtubules of *Leishmania mexicana amazonensis*. The arrow points to a profile of the endoplasmic reticulum eventually inserting between the regularly spaced microtubules (from Pimenta and De Souza 1985 with permission)

are inserted between previously existing ones in such a way that the distance between the microtubules remains constant. This implies the existence of a mechanism that controls the assembly of new microtubules in close connection with increases in the diameter of the cell, probably in response to the insertion of new plasma membrane components.

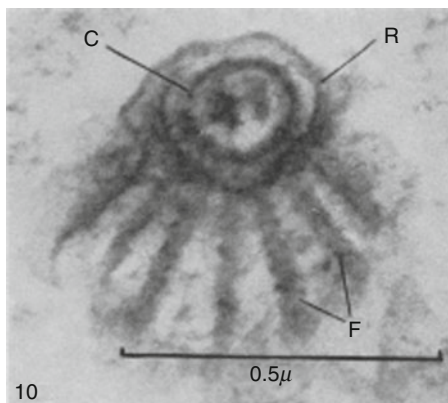
As in other organisms, α/β -tubulin heterodimers are the major proteins found in subpellicular microtubules (Bordier et al. 1982; Stieger et al. 1984). COOH-terminal tyrosinilation of α -tubulin has been shown to take place in *T. brucei* (Stieger et al. 1984). In these organisms, the tubulin genes are arranged in a cluster or 13–18 tandemly repeated alternating α/β pairs, although variations may occur (Reviewed in Kohl and Gull 1998).

In the Bodonina suborder, besides the presence of two flagella and a conspicuous set of bended microtubules that constitute the preoral ridge, a set of subpellicular microtubules is also present. However, as observed in detergent-extracted and critical point-dried cells, subpellicular microtubules in these organisms only partially encircle the cell body, forming two subsets of 22-nm thick microtubules: one that partially surrounds the cell body, and another that remains attached to the recurrent flagellum after extraction (Attias et al. 1996).

2.2 In Apicomplexans

Subpellicular microtubules of Apicomplexa were observed for the first time as early as 1942 by Emmel et al. in *Plasmodium*. Then, Bringmann and Holz (1953) prepared whole mounts of lysed parasites in which subpellicular microtubules could be visualized by electron microscopy. Another attempt to study the ultrastructure of *T. gondii* with a transmission electron microscope was made by Meyer and Andrade Mendonça (1955). At that time, ultrathin sectioning was not available and whole tachyzoites fixed with osmium tetroxide were too thick to

Fig. 3 Ultrathin section of the apical portion of *Toxoplasma gondii* showing the subpellicular microtubules (F) arising from the apical polar ring (R) and, inside it, the conoid (c) (from Garnham et al. 1962 with permission)



allow observation of the inner structure. Later on, in 1957, the same authors published the first electron micrographs of ultrathin sections of *T. gondii* in tissue cultures, but the first mention of subpellicular microtubules, then called “peripheral fibrils,” is found in a paper by Garnham et al. (1962) (Fig. 3). “Surface fibrils” were also described by McLaren and Paget (1968) in *Eimeria tenella* and by Sheffield and Melton (1968) in *T. gondii*. The role of the subpellicular microtubules in maintaining the shape of these protists was first proposed by Kikkawa and Gueft (1964). A few years later, in 1972, several publications already made references to the subpellicular microtubules of *T. gondii*, based on observations made on thin sections (Jones et al. 1972; Vivier and Petitprez 1972) or by negative staining of tachyzoites (De Souza 1972).

Apicomplexa zoites lack microtubule-built locomotory structures such as flagella, although these are found in microgametocytes. Despite this absence of flagella, zoites of the Apicomplexa phylum – merozoites, trophozoites, tachyzoites, bradizoites, and sporozoites – are highly motile and rapidly penetrate into host cells. Active invasion depends on parasite motility and the sequential secretion of products from the micronemes and rhoptries, club-shaped secretory organelles that discharge their products through the apical portion of the parasite. Sequential secretion by micronemes and rhoptries and the coordinated movement of the conoid promote the attachment of the infective form to the surface of the host cell and the subsequent invasion of the host cell, where the parasite multiplies within a parasitophorous vacuole (Reviews in De Souza 2006; Ravindran and Boothroyd 2008).

3 Visualization of the Whole Network of Subpellicular Microtubules

Only by electron microscopy techniques, it became possible to observe subpellicular microtubules. However, very early, before thin sectioning and efficient chemical fixation became available, it was possible to observe them in ruptured

or detergent-extracted samples negatively stained. Soon, its presence was described in all genera and evolutive forms of Trypanosomatids and Apicomplexans.

3.1 In Trypanosomatids

Since the first observations of thin sections, it became clear that subpellicular microtubules are present throughout the trypanosomatid cell body except at the flagellar pocket region, where at one point the microtubule corset is replaced by a flagellum attachment zone (FAZ) formed by a filamentous structure. This structure is also associated with a group of three to four microtubules that are closely associated with the cisternae of the endoplasmic reticulum and originate close to the basal bodies. These microtubules differ from subpellicular microtubules because they are not depolymerized when the cells are incubated in a medium with high salt concentrations (Sherwin and Gull 1989). Additionally, they are the only microtubules stained with the monoclonal antibody 1B41, which recognizes β -tubulin (Gallo and Precigout 1988). These subpellicular microtubules are also found in structures such as the cytostome–cytopharynx complex, a deep invagination of the plasma membrane that may reach the proximities of the nuclear region.

Pioneering attempts to observe the distribution of subpellicular microtubules in whole cells were made by Angelopoulos (1970), who ruptured protozoan cells using surface tension spreading followed by critical point drying (Fig. 4). The second attempt was carried out by De Souza and Benchimol (1984), whose method consisted of adhering the protozoan to a formvar/carbon-coated grid previously coated with poly-L-lysine, followed by a light extraction with Triton-X 100, critical point drying, and observation under a high-voltage electron microscope (1,000 KV) installed at the University of Colorado, Boulder. The extraction of the membrane did not interfere with the general shape of the cell and maintained the integrity of the subpellicular microtubules (De Souza and Benchimol 1984). The third attempt was by Gull and coworkers, who used negative staining to obtain a sharp visualization of microtubule arrangement (Fig. 5) in *Trypanosoma brucei* (Gull 1999). A fourth approach used field emission scanning electron microscopy of detergent-extracted cells (Fig. 6). It is important to point out that none of these methods revealed the presence of a typical microtubule-nucleating center from which the microtubules emerge.

3.2 In Apicomplexans

In Apicomplexans, the presence of microtubules located below the inner membrane complex was noticed since the first observations made in thin sections of well-preserved tachyzoites of *T. gondii* (Garnham et al. 1962; Sheffield and Melton 1968). However, a better view of the extension of this system was obtained when

Fig. 4 Distribution of the subpellicular microtubules in whole cells of *Crithidia fasciculata* ruptured and critical point dried. (mc) microtubule curve, (f) flagellum, (pa) posterior apex. Bar: 1 μ m (from Angelopoulos 1970 with permission)

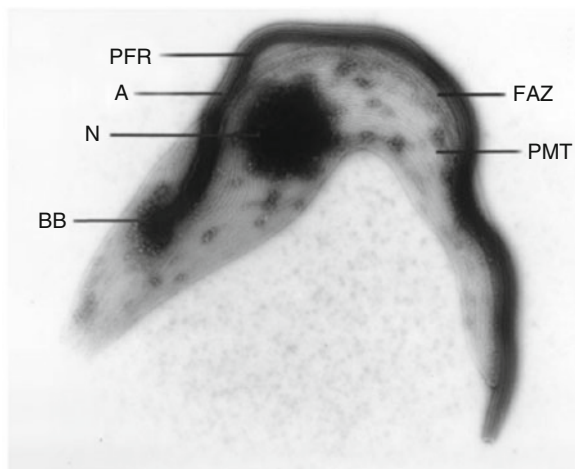


Fig. 5 Negative staining of *Trypanosoma brucei* cytoskeleton after detergent extraction. (FAZ) flagellar attachment zone, (BB) basal body, (PFR) paraflagellar rod, (PMT) subpellicular microtubules, (A) axonema and (N) nucleus (from Gull 1999, with permission)

the whole tachyzoite was slightly disrupted by rinsing with distilled water, deposited on formvar- and carbon-coated grids, negatively stained with phosphotungstic acid, and observed under an electron microscope (Fig. 7) (De Souza 1972;

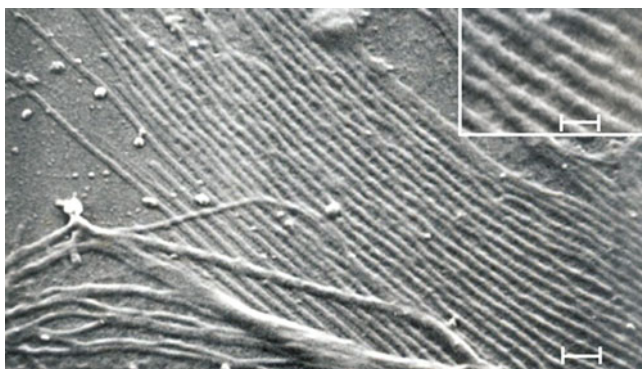


Fig. 6 Subpellicular microtubules of a kinetoplastid observed by field emission scanning electron microscopy after detergent extraction and critical point drying. Spacing of microtubules of various lengths is kept by regularly spaced bridges. *Bar* = 300 nm. *Inset* = 600 nm

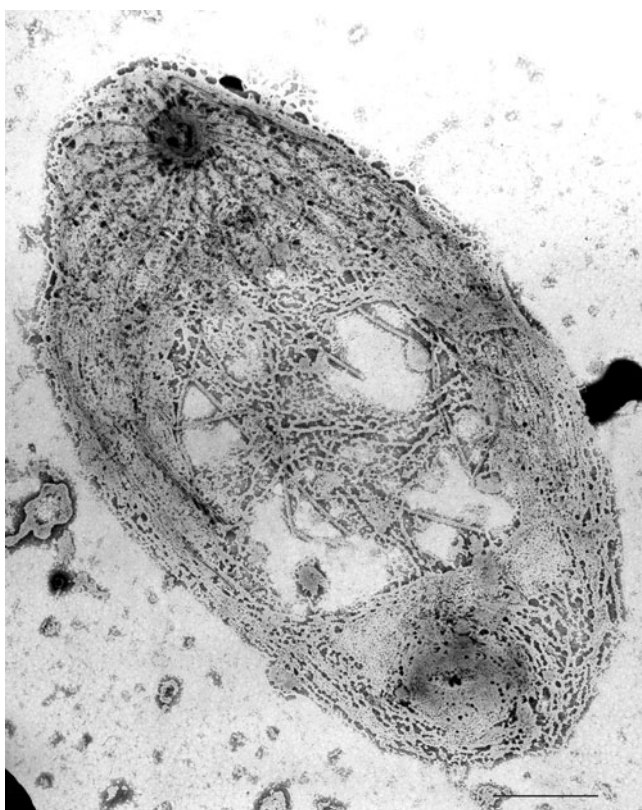
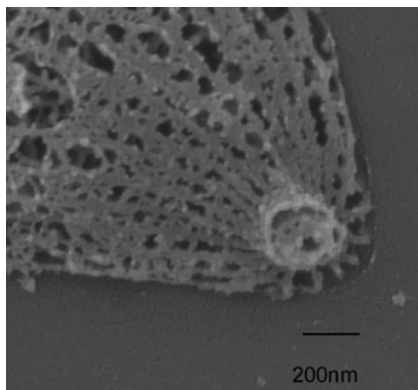


Fig. 7 Negative staining of a tachyzoite after membrane extraction. The 22 subpellicular microtubules around the posterior polar ring can be individualized. The central pair of microtubules is shown by the *white arrowhead*. *Bar* = 1 μ m

Vivier and Petitprez 1972), as previously done with sporozoites of *Eimeria ninakohlyakimovae* (Roberts and Hammond 1970). Using this simple approach, it was shown that the subpellicular network remains intact and that the microtubules radiate from a polar ring located at the base of the conoid, a structure also formed by microtubules (De Souza 1972, 1974). Two short intraconoidal microtubules and 22 subpellicular microtubules were observed in all *T. gondii* tachyzoites. In addition, it was shown that the microtubules ended freely, reaching about 70% of the cell length toward the posterior region of the cell. These initial observations were confirmed and extended to other members of the Apicomplexa phylum, with some differences in the number of subpellicular microtubules. For instance, 24 microtubules were counted in both *E. ninakohlyakimovae* (Roberts and Hammond 1970) and *Eimeria acervulina* (Russel and Burns 1984), 15–16 in *Plasmodium berghei* sporozoites (Vanderberg et al. 1967), and 22 in *Sarcocystis neurona* (Speer and Dubey 2001). Subpellicular microtubules are usually evenly distributed around the apical ring, but in *P. falciparum* they form a band instead. It was later shown that the basal polar ring constitutes a microtubule-organizing center (MTOC) from which the microtubules originate, projecting toward the posterior region, which corresponds to the plus end where new tubulin dimers are added for microtubule growth (Russel and Burns 1984). Several genera of Apicomplexa have two other structures constituted by tubulin: the conoid and the microtubules that constitute the mitotic apparatus (Review in Morrisette and Sibley 2002); the conoid will be considered further in this chapter but the mitotic spindle is not within the scope of the present review. Field emission scanning electron microscopy of *T. gondii* tachyzoites, whose membrane pellicle was extracted with detergent before fixation, corroborates the uniform distribution of subpellicular microtubules around the polar ring (Fig. 8). In its resting position, the conoid lies below the polar ring and is surrounded by subpellicular microtubules, making its observation rather difficult; however, in the extruded state, it rises above the polar ring, and the helical assembly of its microtubules can be observed.

Fig. 8 Field emission scanning electron micrograph of the anterior portion of *Toxoplasma gondii* after extraction with detergent and critical point drying. The conoid arises from the inner side of the polar ring to which subpellicular microtubules are attached. Bar: 200 nm



4 High-Resolution Images of the Microtubules

High-resolution images of microtubules were reported as early as in the 1970s. However, in recent years, improvements both in fixation techniques, as freeze-substitution, and in microscopic resources, as electron tomography, brought new insights and information into the constitution of these structures, revealing peculiarities that are neither found in higher eukaryotes nor shared between Trypanosomatids and Apicomplexans.

4.1 In Trypanosomatids

Four approaches were used to obtain high-resolution images of subpellicular microtubules. The first approach was based on previous results by Mizuhira and Futeasaku (1972), who showed that the addition of tannic acid to the glutaraldehyde solution significantly improved the preservation of the microtubules. In addition, some microtubules appeared as though they had been negatively stained so that their internal structure could be visualized. Using this technique, it was observed that the subpellicular microtubules of trypanosomatids are made up of 13 typical protofilaments (Soares and De Souza 1977) (Fig. 9). However, this technique did not reveal further details on the associations of the microtubules with each other or with the plasma membrane. The second approach used quick-freezing, followed by freeze-fracture, deep-etching, and rotary replication (Souto-Padron et al. 1984), resulting in well-preserved microtubules. Indeed, longitudinal views of the microtubules clearly revealed their internal structures (Fig. 10) and showed the presence of filaments that connect the microtubules to each other. It was also clear that these connections are regularly spaced. In addition, the connections of the microtubules with the plasma membrane, the endoplasmic reticulum, and the reservosomes were all visualized. We still do not know the nature of these filaments. Although proteins associated with subpellicular microtubules have been identified, we still have not elucidated a clear topological relationship between structure and composition. The third approach analyzed microtubule structure by optical diffraction and revealed a spacing of 5 nm between protofilaments and a 4-nm axial periodicity corresponding to the tubulin subunits, which were also in freeze-etching replicas. The pitch of the shallow left-hand three-start helix is 12° . The fourth approach used the fracture-flip technique, allowing the visualization, at high resolution, of the actual inner surface of the *Leishmania* plasma membrane (Fig. 11). The subpellicular microtubules were seen clearly. Treatment of the sample with trypsin led to the disappearance of the microtubules, leaving behind parallel arrays of particles that may correspond to proteins that link the microtubules to the plasma membrane (Hou et al. 1992).

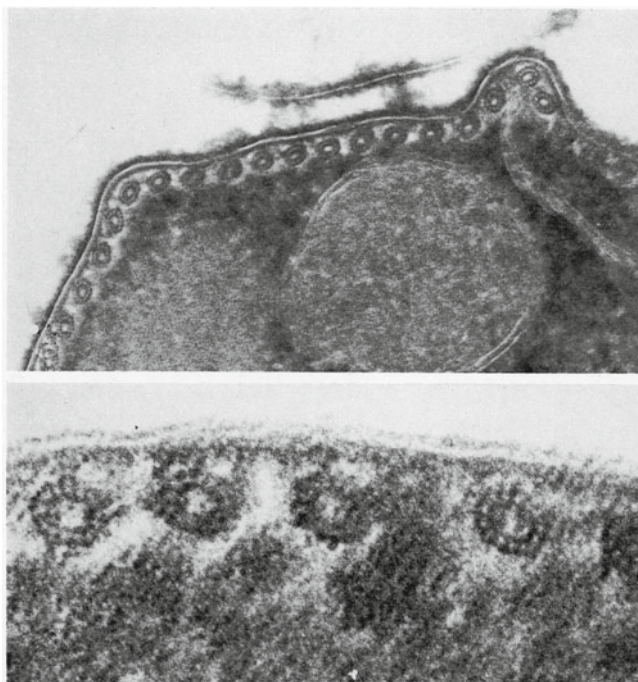


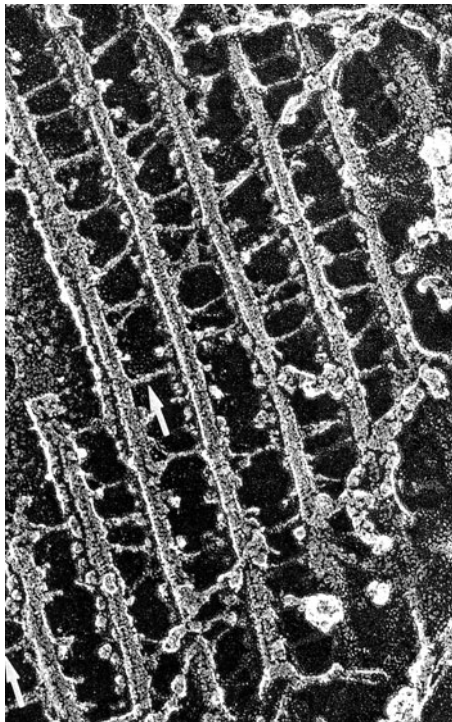
Fig. 9 Cross-section of subpellicular microtubules of *Leptomonas samueli* fixed with glutaraldehyde and tannic acid. (a) General view of the assembly of microtubules under the plasma membrane. (b) The 13 tubulin subunits of each microtubule can be counted, as well as connections between the microtubules and the plasma membrane. Bars: (a) 100 nm, (b) 20 nm (From Soares and De Souza 1977 with permission)

4.2 In Apicomplexa

As mentioned above, the subpellicular microtubules of Apicomplexa show striations along their length (Fig. 12) (De Souza 1974). Such striations were better visualized following the application of Fourier analysis techniques to the images of isolated, frozen-hydrated microtubules in which a 32-nm periodicity was observed (Fig. 13) (Morrisette et al. 1997). This suggested that the subpellicular microtubules are decorated along their length with a MAP that could be responsible for their great stability under high pressure, cold and treatment with detergents typically used to isolate them. A similar periodicity was also observed in rows of intramembranous particles observed in freeze-fracture replicas of the inner pellicular membrane of *Toxoplasma* that overlaid the subpellicular microtubules (Fig. 14). That was the first undisputable evidence that subpellicular microtubules are linked to the pellicle and could be associated with parasite motility.

It is interesting to point out that Russel and Burns (1984) had been able to see that subpellicular microtubules interact side-on, rather than end-on, with the polar

Fig. 10 Replica of quick-freeze, deep-etched, rotary-shadowed epimastigote form of *Trypanosoma cruzi* showing the subpellicular microtubules and the filamentous bridges connecting them to each other side by side



ring, an observation corroborated with the use of cryo-electron tomography (Cyrklaff et al. 2007) (Fig. 15). Besides confirming the lateral association between subpellicular microtubules and the polar ring, cryoelectron tomography revealed that the microtubules on the cell walls of *Plasmodium berghei* sporozoites are extended at the luminal side by an additional 3 nm in comparison to the microtubules of mammalian cells (Fig. 15). Furthermore, Fourier analysis revealed an 8-nm longitudinal periodicity of the luminal constituent, suggesting the presence of a molecule interacting with tubulin dimers, probably stabilizing them. Subpellicular microtubules of *T. gondii* tachyzoites shared the same features but microtubules from pig brain cells did not, indicating a conserved pattern among Apicomplexans.

5 Immunocytochemical Characterization of the Microtubules

Immunocytochemistry arrived as a powerful tool for the identification and localization of several components of the cell. It has been largely applied both for the identification of subtypes of tubulin and for the localization of other proteins associated with it that participate in the maintenance of the assembly and motion of the complex.

Fig. 11 The inner surface of the plasma membrane of *Leishmania major* as well as of a portion of the flagellum (asterisk) showing the subpellicular microtubules as revealed by the fracture-flip technique. Bar, 250 nm (from Hou et al. 1992)

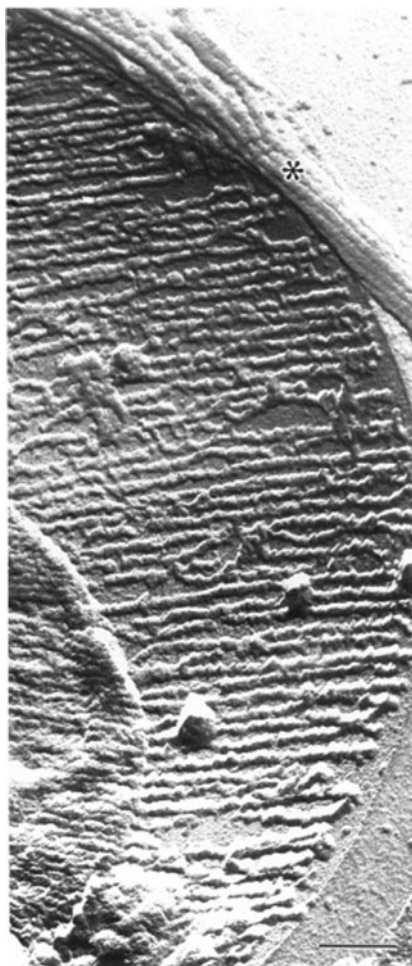
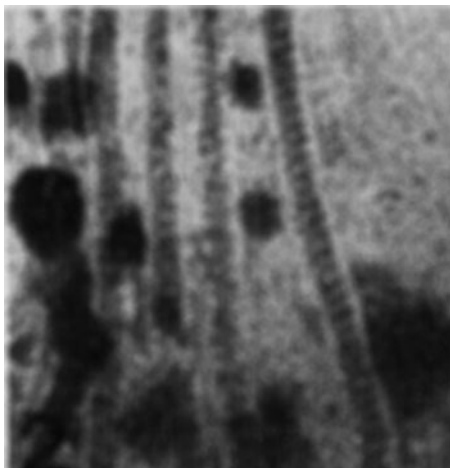


Fig. 12 Striations along the subpellicular microtubules of *Toxoplasma gondii* observed in negatively stained tachyzoites (from De Souza 1974 with permission)



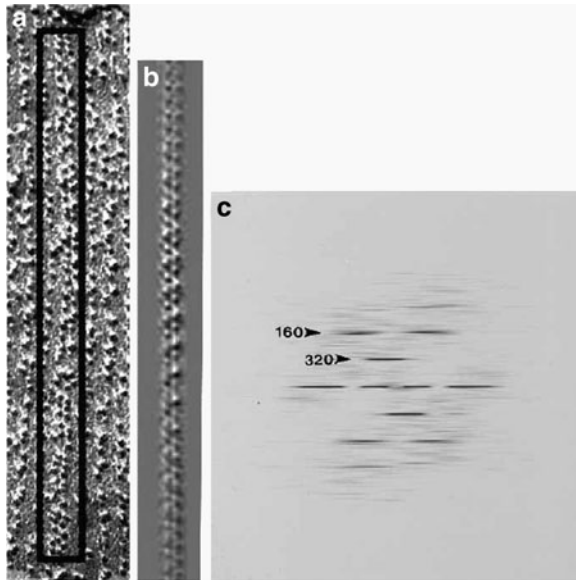
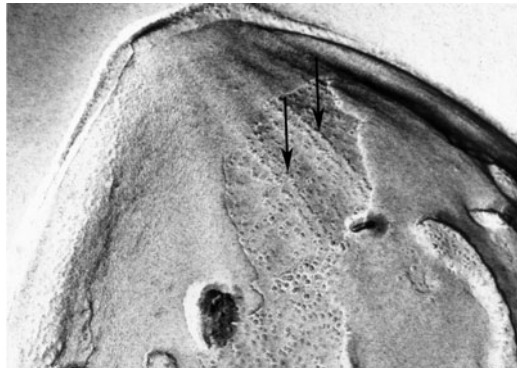


Fig. 13 Analysis of longitudinal double particle rows from freeze-fractured samples (a) provides a calculated diffraction pattern (C), which exhibits a pronounced 320 Å periodicity, corresponding to alternate particles in the rows. (b) A line-filtered reconstruction of the double particle rows, generated from information found on the strong layer lines. Note that the particles in adjacent rows are pitched relative to row length. When areas of the fracture face containing both single and double rows of IMPs are used to generate computed diffraction patterns (from Morrisette et al. 1997 with permission)

Fig. 14 Freeze-fracture of *Toxoplasma gondii* tachyzoites. P face of the inner pellicular membrane showing the double rows of intramembranous particles positioned according to the subpellicular microtubules (from Cintra and De Souza, 1985a, with permission)



5.1 In Trypanosomatids

Monoclonal antibodies that recognize several tubulin isotypes have been used to characterize the subpellicular microtubules of trypanosomatids. Multiple tubulin genes exist in trypanosomes (Seebeck and Gehr 1983) but the mRNAs of only one

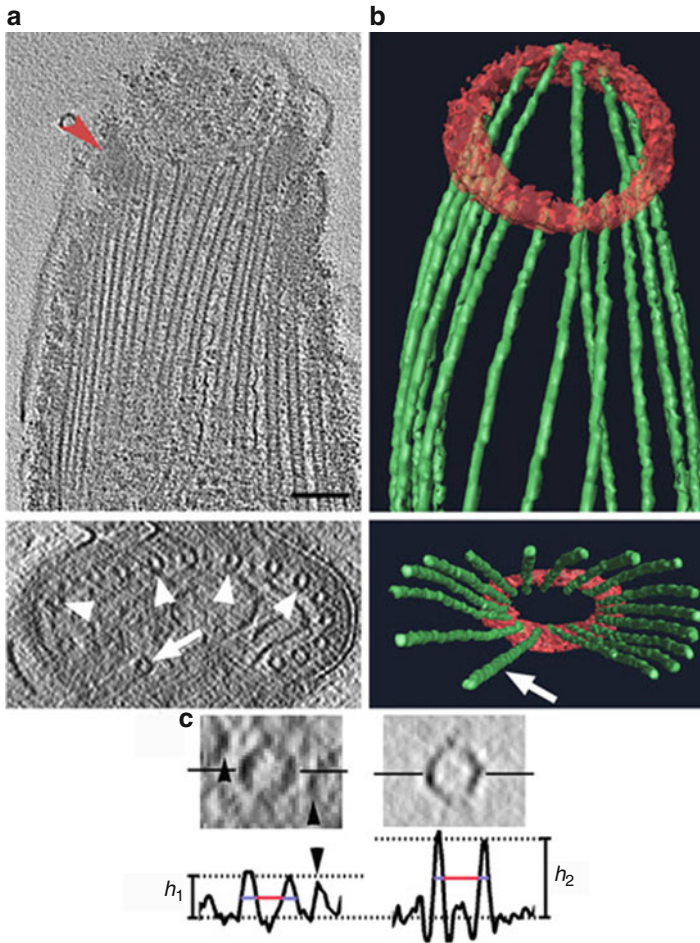


Fig. 15 (a) Longitudinal (top) and cross (bottom) slices through a tomographic reconstruction from the apical part of a *Plasmodium* sporozoite revealing subpellicular microtubules. Red arrowhead, polar ring; white arrowheads, circumferentially arranged microtubules; arrow, the lone microtubule. (b) Volume-rendered representations of the microtubules (green) and the polar ring (red) from the same tomogram. (c) Cross-sections of microtubules from a sporozoite (left) and from in vitro polymerization (right). Arrowheads indicate neighboring microtubules. Density distributions along the horizontal lines are displayed in the graphs. Dotted lines indicate background and maximal heights (h_1 and h_2). Microtubule widths (blue-red lines) were determined at half-heights. Red line, diameter of lumen; blue lines, microtubule walls. Bar, 100 nm (from Cyrklaff et al. 2007, with permission)

type of α -tubulin and one type of β -tubulin have been detected; however, posttranslational modifications such as acetylation, glutamylation, and detyrosination take place (Stieger et al. 1984; Sasse and Gull 1988; Schneider et al. 1997; Westermann et al. 1999). Antibodies recognizing acetylated tubulin, considered by several

authors as characteristic of stable microtubules, label subpellicular as well as flagellar microtubules (Sasse and Gull 1988; Souto-Padron et al. 1993). However, acetylated tubulin was also detected in *T. brucei* spindle microtubules, which are labile structures (Sasse and Gull 1988). The use of an antibody recognizing tyrosinated α -tubulin showed that polymerizing microtubules have a high content of tyrosinated tubulin that is subsequently lowered to a basal level. These observations led to the suggestion that this system may provide the cells with a mechanism to discriminate between new and old microtubules (Sasse and Gull 1988). Through the use of several antibodies, it has also been shown that γ -tubulin, usually found in microtubule-organizing centers such as basal bodies, seems to exist in a small subset of subpellicular microtubules (Scott et al. 1997; Libusova et al. 2004).

Robinson et al. (1995) analyzed in detail the polarity of the subpellicular microtubules of *T. brucei*. Using the hook decoration technique, in which detergent-extracted cytoskeletons are incubated in the presence of exogenous tubulin, they observed the decoration of the subpellicular microtubules. In cytoskeletons viewed from the posterior toward the anterior region of the protozoan (relative to the basal bodies and flagellum), 93% of the decorated microtubules had a clockwise hook curvature, thus indicating that the plus end of the microtubules (sites where microtubule growth takes place) is at the posterior region of the cell. The same authors also used the negative staining technique to test polarity by observing points of microtubule growth when the cytoskeleton was incubated in the presence of tubulin. Again, growth was observed in the posterior region. In addition, they took advantage of previous observations that indicated that tyrosinated α -tubulin is a marker for newly assembled microtubules (Kohl and Gull 1998) and incubated the cytoskeleton with the YL 1/2 monoclonal antibody, which recognizes tyrosinated tubulin. Areas of labeling were concentrated in the posterior third of the protozoan cell (Robinson et al. 1995). Another modification found in some microtubules is the presence of tubulin polyglycylation, as occurs in *Giardia lamblia* (Weber et al. 1996; Campanati et al. 2003). No such posttranslational modification of tubulin was found in trypanosomatids (Schneider et al. 1987).

One intriguing question is how the number of microtubules increases during cell division. Available data indicate that the addition of new microtubules into the layer takes place by intercalation between the existing microtubules (Sherwin and Gull 1989). Drugs that interfere with microtubules block the process of cytokinesis, giving rise to cells whose diameter does not reach the values found in control cells; this then triggers the invagination of the plasma membrane so that two new cells are formed (De Souza unpublished observations).

5.2 In Apicomplexa

Apicomplexans were shown to have unlinked single copies of genes for α -, β -, and γ -tubulins (Reviewed in Morrisette and Sibley 2002). Both *Plasmodium yoellii* and *P. falciparum* were shown to have two independent genes for α -tubulin (I and II),

with the α -tubulin II gene being specifically expressed in the flagella of male gametes (Rawlings et al. 1992). α -tubulin II is a male-specific protein in *P. falciparum*.

Employing the technique of “hook decoration” of *E. acervulina* subpellicular microtubules with tubulin extracted from pig brains, Russel and Burns (1984) demonstrated that the subpellicular microtubules of Apicomplexa grow from the polar ring, a unique microtubule-organizing center, with their plus ends toward posterior end of the parasite. So far, γ -tubulin has not been shown to be associated with the polar ring.

6 Biochemistry

The biochemical nature of several proteins associated with the subpellicular microtubules in Trypanosomatids and their putative roles have been identified. However, the same is not true in what concerns Apicomplexans, may be for the limitations associated with the impossibility of obtention of axenic cultures of any stage.

6.1 In Trypanosomatids

We have very little information about the nature of the filaments that connect the subpellicular microtubules of trypanosomatids to each other, to the plasma membrane or to cytoplasmic organelles. As shown in Fig. 2, there is a clear connection between the microtubules and the endoplasmic reticulum, which on some occasions penetrates between the microtubules (Pimenta and De Souza 1985). One of the first suggestions pointed to the possible presence of dynein as a microtubule-associated protein. This hypothesis has not been confirmed.

Biochemical analysis of cytoskeleton preparations, mostly obtained by detergent extraction, pointed to the presence of several proteins associated in some way with the microtubules. First, a 28-kDa protein was identified and localized only in the posterior regions of the cell. This protein, designated as Gb4, is encoded by a large gene consisting of numerous repeated units of 0.6 kb linked (Rindisbacher et al. 1993). Second, there is a family of high-molecular weight proteins that consist of more than 50 nearly identical tandemly repeated highly conserved 38-amino acid units (Affolter et al. 1994; Schneider et al. 1988). One of the proteins, known as MARP-1 (microtubule-associated repetitive protein), was isolated and was shown in vitro to bind to tubulin at sites different from those involved in the binding of the brain MAPs Tau and MAP2 (Hemphill et al. 1992). Under immunofluorescence microscopy, part of the protein in mammalian cells was observed binding to the microtubules. Third, Balaban et al. (1989) isolated the subpellicular microtubules of *T. brucei* using a high-ionic strength salt solution, and identified two peaks of 52 and 53 kDa. The first one corresponded to a tubulin-binding protein. Experiments of in vitro microtubule assembly using brain tubulin showed that, in the presence of the 52-kDa protein, the assembled microtubules formed bundles with regular (7 nm,

center-to-center) cross-links. Subsequently, the same group reported the presence of a 15-kDa protein that promotes the bundle formation of microtubules assembled *in vitro* and is localized along the subpellicular microtubules (Balaban and Goldman 1992). Fourth, Thomas et al. (2000) described the kinetoplastid membrane protein-11 (KMP11) and showed its downregulation in parasites treated with vinblastine. Fifth, Vedrenne et al. (2002) found two related low-molecular weight cytoskeleton-associated proteins, designated as CAP15 and CAP17, in the cytoskeleton of *T. brucei*. Immunocytochemistry observations showed labeling associated with the subpellicular microtubules, especially in the anterior region of the protozoan cell. However, the resolution was not sufficient to determine the exact localization of these two proteins. Again, mammalian cells transfected with a CAP15 plasmid labeled the microtubules, thus confirming that it is a microtubule-associated protein. Sixth, Schneider et al. (1988) reported the presence of a microtubule-binding protein, designated as p41, that carries covalently bound fatty acids. The association of this protein with the microtubules was dependent on the presence of Ca, as it was released when incubation was carried out in the presence of EGTA. Seventh, Kratzerová et al. (2001) used a monoclonal antibody (MA-01) to describe a 210-kDa microtubule-interacting protein that labels the subpellicular microtubules located at the posterior region of *Leishmania* promastigotes. Moreover, this antibody also labeled the flagellum and the mitotic spindle. Eighth, a 60-kDa protein with 10–12 transmembrane domains, designated as NRAMP1, was found to associate with the microtubules as well as with the plasma membrane, thus suggesting that it is involved in the association between these two structures (Kishi et al. 1996). Ninth, a 210-kDa protein detected using a monoclonal antibody was closely associated with the cross-bridges lying between the microtubules (Woods et al. 1992). Finally, and more recently, Baines and Gull (2008) described a highly phosphorylated protein, designated as WCB, recognized by a monoclonal antibody. This protein presents an N-terminal C2 domain characteristic of membrane-associated proteins and a repetitive, charged C-terminal region with the characteristics of a microtubule-binding domain. However, no high-resolution localization of the protein has been reported. With RNA interference depletion of WCB, it was shown that this protein is essential for cell morphogenesis.

7 Drug Sensitivity

Causative agents of several diseases of great impact on human and veterinary health belong to the phylum Apicomplexa – Plasmodium, Toxoplasma, Eimeria. The same is true for Trypanosomatids that include the agents of Chaga's disease, African Trypanosomiasis, and Leishmaniasis. Most of these diseases can be efficiently treated and cured, so there is a permanent concern about finding new drugs for their treatment. In this sense, exclusive organelles such as the apicoplast of Apicomplexa, the glycosomes of Trypanosomatids, and metabolic pathways that are not shared by the hosts are targets with a great potential.

7.1 *In Trypanosomatids*

The sensitivity of the subpellicular microtubules of trypanosomatids to disrupting agents is very particular. Most microtubules are sensitive to low temperature, high pressure, and several drugs. For instance, drugs such as colchicine and vinblastine efficiently depolymerize the cytoplasmic microtubules of mammalian cells. However, in the case of trypanosomatids, some of these agents do not interfere with the subpellicular microtubules (Filho et al. 1978; Grellier et al. 1999). In contrast, drugs such as oryzalin and trifluralin (Chan and Fong 1994), which have no effect on mammalian microtubules, are potent disruptors of the trypanosomatid cytoskeleton (Seebeck and Gehr 1983; Chan et al. 1991; Bogitsh et al. 1999). Phenothiazines are also highly effective against subpellicular microtubules (Seebeck and Gehr 1983). On the other hand, drugs such as Taxol, which stabilize microtubules, induced significant morphological changes in the tested trypanosomatids (Baum et al. 1981; Webovetz et al. 2003; Dantas et al. 2003; Havens et al. 2000; Kapoor et al. 1999). All this information, in association with the fact that subpellicular microtubules remain intact throughout the cell cycle, clearly indicates that these are not classical microtubules as described in mammalian cells.

7.2 *In Apicomplexa*

Studies using classical drugs that disrupt the microtubules found in mammalian cells, such as colchicine and vinblastine, showed that the subpellicular microtubules of Apicomplexan protozoa are highly resistant to them. Only at high concentrations (1 mM colchicine) was the shortening of the microtubules observed. However, the microtubules were markedly sensitive to dinitroanilines (trifluralin, oryzalin, and ethafluralin), which interfere with plant microtubules (Stokkermans et al. 1996). This resistance is associated with point mutations in α -tubulin, especially in the M or N loops, which coordinate protofilament interaction in the microtubules and in the core of the α -tubulin (Morrisette et al. 2004; Ma et al. 2007).

8 Microtubule–Microtubule and Microtubule–Plasma Membrane Associations

The regular spacing between each microtubule, and between microtubules and the pellicle, is maintained by linker proteins, most of them still unknown at this time. Proteomic analysis is being currently applied and should quickly broaden the knowledge about them.

8.1 In Trypanosomatids

Electron microscopy analysis carried out to evaluate the isolation of subcellular fractions of trypanosomatids showed that even after disruption of the cells the microtubules remained attached to portions of the plasma membrane (Fig. 16) (De Souza 1976; Hunt and Ellar 1974; Dwyer 1980). Subsequently, several methodologies were developed to isolate membrane fractions. In some protocols, the microtubules remained attached and were even used as a morphological criterion to assess the purity of the fraction. In others, however, the microtubules disappeared (Reviewed in De Souza and Cunha e Silva 2003). More recently, a detailed proteomic analysis of isolated plasma membrane sheets with associated microtubules was carried out in the bloodstream forms of *T. brucei* (Bridges et al. 2008). The authors inferred which proteins were associated with the plasma membrane and which were associated with the cytoskeleton. A large number of proteins were identified as belonging to the cytoskeleton. However, no attempts were made to localize these proteins.

8.2 In Apicomplexa

Connections between subpellicular microtubules and the inner membrane complex have been observed in detergent-extracted *T. gondii*, especially when tannic acid was added to the glutaraldehyde solution used to fix the cells before processing for observation by transmission electron microscopy (Cintra and De Souza 1985b). The connections were regularly spaced at intervals of about 30 nm (Fig. 17).

The use of the freeze-fracture technique revealed the presence of a parallel array of closely apposed intramembranous particles on the P fracture face of the inner membrane complex. The distance between each particle strand was 28 nm, which corresponded to the diameter of the microtubules (Dubremetz and Torpier 1978;

Fig. 16 Subpellicular microtubules remain associated with the plasma membrane even after the disruption of epimastigote forms of *Trypanosoma cruzi* (from De Souza 1976 with permission)

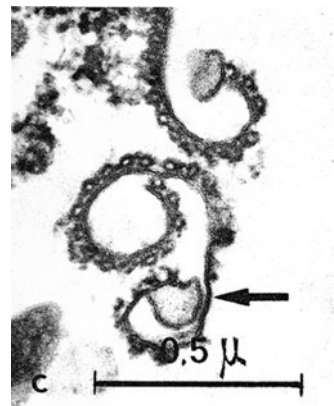
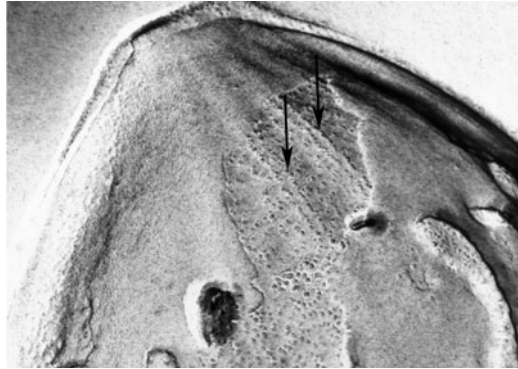


Fig. 17 Tachyzoites of *Toxoplasma gondii* fixed with glutaraldehyde and tannic acid after detergent extraction. Cytoplasm and most of the outer membranes (om) were extracted exposing regularly spaced bridges between the subpellicular microtubules and the inner pellicular membrane (from Cintra and De Souza 1985b with permission)



Porchet and Torpier 1977; Cintra and De Souza 1985a). Similar images were also observed on the fracture faces of the intermediate membrane (Cintra and De Souza 1985a) (Fig. 14). Application of Fourier analysis techniques better revealed this structure, with a 32-nm repeat in the double rows of membrane particles. Drugs known to disrupt cytoskeletal components fail to destroy the integrity of the particle lattice (Morrisette et al. 1997). Although there are no data on the nature of the particles that make up these strands, it is possible that they result from some linkage of the subpellicular microtubules to proteins located in the inner and intermediate membranes via filamentous structures that are not yet identified.

Unlike the subpellicular microtubules of Trypanosomatids, those of Apicomplexans are linked by the minus end to the polar ring, but no lateral bridges connect them laterally. Instead, a subpellicular network of filaments, which are easily observed in tachyzoites following detergent extraction and negative staining, runs parallel to the microtubules (Mann and Beckers 2001). Two proteins were identified in this network: TgIMC1 and TgIMC2. TgIMC1 is similar to articulins, the major cytoskeletal proteins of free-living protists and algae. In *P. falciparum*, a homolog of TgIMC1 with an additional 220 amino acids was identified (Khater et al. 2004). The presence and distribution of plateins, a subfamily of articulins, in *T. gondii* tachyzoites was demonstrated by immunofluorescence by Lemgruber et al. (2009). A diffuse labeling of the whole cell body was observed and electron microscopy of detergent-extracted tachyzoites exposed a network of 10-nm filaments distributed throughout the parasite and labeled by antiplatein antibodies. A predicted IMC protein was identified in the *T. gondii* genome; its sequence had 25% identity and 42% similarity with the platein isoform alpha 1, described in *Euplotes aediculatus*, and 42% identity and 55% similarity with the *Euglena gracilis* homolog, denoting its resemblance to articulins.

8.3 The Conoid in Apicomplexa

As already stated, among the characteristics that define the Apicomplexa, the apical complex is so outstanding that the whole phylum was named after it. In addition to

the secretory organelles that discharge their contents through the apical complex, this unique structure is formed by a posterior polar ring to which the subpellicular microtubules attach; it also has two apical rings and a conoid, a hollow cone-shaped structure formed by microtubules in a particular spring-like spiral arrangement, and two intraconoidal microtubules that are shorter than the subpellicular microtubules and may serve to deliver secretory vesicles that participate in host cell invasion (Nichols and Chiappino 1987; Carruthers and Sibley 1997). Early descriptions of the conoid were provided by several authors (Gustafson et al. 1954; McLaren and Paget 1968; Sheffield and Melton 1968; Vivier and Petitprez 1972; de Souza 1974) and its general structural organization was reviewed by Attias and De Souza (in press).

The conoid is not present in all Apicomplexans; for instance, *Plasmodium* spp. lack this structure in their apical complex. On the other hand, it is present in *Toxoplasma*, *Eimeria*, *Gregarina*, and *Sarcocystis*. In *Toxoplasma*, a fine description that included an estimation of the diameter (300–350 nm) and number of fibers (15–23) of the conoid was made by Vivier and Petitprez (1972). By comparing the distances between the polar ring and the conoidal rings as observed by negative staining, De Souza (1974) suggested that the conoid moves up and down (Figs. 4 and 5 from De Souza 1974). The central pair of microtubules inside the conoid was also described in these early papers, but its function and relation to the other conoidal structures remain unknown. Figure 18 depicts the current model of structural organization of the conoid (Attias and De Souza in press; Dubey et al. 1998; Hu et al. 2006). The most consistent contribution on the structural organization of the conoid was provided by Hu et al. (2002), who showed that each conoidal fiber extends by about half of the diameter of the conoid and that there are about six fibers in longitudinal cross-section, confirming the early reports by Vivier and Petitprez (1972). Furthermore, by using transgenic *T. gondii* expressing YFP fused to the N-terminus of tubulin, Hu et al. (2002) confirmed that it is the main structural component of the conoidal spiral fibers. However, labeling of α - and β -tubulin by monoclonal antibodies required vigorous detergent extraction combined with high salt concentrations. Thin sections of parasites fixed in the presence of tannic acid revealed that each of the microtubules in the central pair is composed by a set of 13 protofilaments, as usual. On the other hand, each of the conoidal fibers has only nine protofilaments arranged in an open conformation that resembles a comma (Hu et al. 2002), a very unusual and unique form of tubulin assembly.

The conoid is not present in all Apicomplexans. However, the posterior polar ring is. As the conoid moves upwards, it protrudes above the posterior polar ring, whereas in the nonprotruding state it is hidden by the polar ring and the subpellicular microtubules that irradiate from it (Nichols and Chiappino 1987; Morrisette and Sibley 2002).

Through observations made on the ultrastructure of the cytoskeleton of *E. acervulina*, Russel and Burns (1984) proposed the posterior polar ring as a MTOC. The main evidence for this polarity was the fact that subpellicular microtubules emanate from the polar ring with their plus ends toward the posterior end of

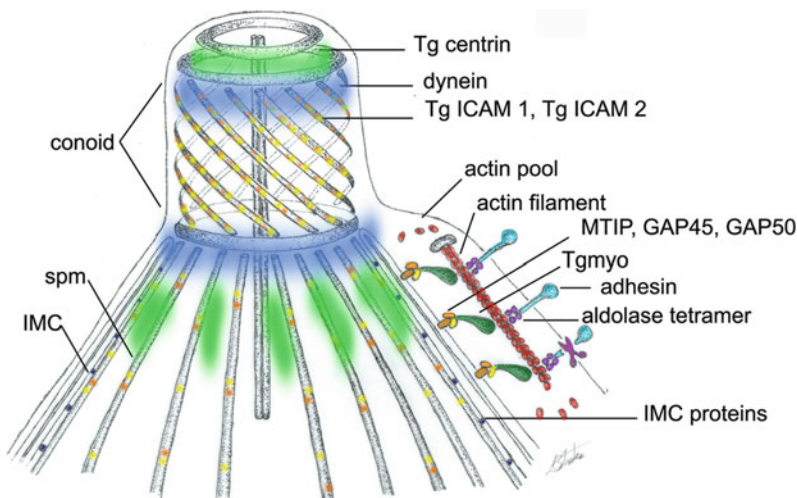


Fig. 18 Scheme of the present knowledge on the molecular and ultrastructural organization on the microtubules and associated proteins in the tachyzoite form of the Apicomplexa *Toxoplasma gondii* summarizing the present knowledge on the structural and molecular organization of the cytoskeleton. Tg centrin, ICAM1 and 2 and Tg centrin have been localized, but its structural interaction with the cytoskeleton has not yet been defined. Tg centrin was localized on the area of the apical polar rings and in patches just under the polar ring, while labeling for dynein was positive at the top and base of the conoid. ICAM 1 and 2 followed the spiral disposition of the conoid fibers, and are sparsely distributed over the subpellicular microtubules (spm). The molecules involved in gliding are represented on the right side of the scheme, where the proportional distance between the plasma membrane and the inner membrane complex (IMC) was disregarded, to allow insertion of all the molecules so far identified (from Attias and De Souza ([in press](#)) with permission)

the parasite, as shown by decoration with exogenous tubulin (Heidemann and McIntosh 1980). These results were corroborated for *T. gondii* by Nichols and Chiappino (1987). Later, the presence of γ -tubulin in the apical portion of *P. falciparum* schizonts was demonstrated by immunofluorescence by Fowler et al. (2001), reinforcing this concept. However, it was not until 2006 that Hu et al. (2006) obtained fractions of *T. gondii* enriched in apical complex cytoskeleton that included the conoid, and identified several of the proteins used to build the conoidal structure. Two of them, TgCAM1 and TgCAM2, have calcium-binding domains and were localized to the conoid area by immunoelectron microscopy. TgDLC is a dynein light chain kinase from *T. gondii* that is 85% identical to human and mouse DLC dynein. It was shown to be present at the spindle poles and in centrioles in addition to the apical portion of the parasites, in which it is distributed above and below the conoid; it is also found on the polar ring at the site of attachment of the subpellicular microtubules. In GFP-DLC-expressing mutants, this protein was also present in the posterior end of the parasites. Two centrin homologs, TgCentrin-2 and TgCentrin-3, were also detected. The first one was predominantly, but not exclusively, found in the conoid-enriched fraction, in the

preconoidal rings and in annular patches in the upper third of the cell, below the polar ring. TgCentrin-3 was faintly localized in the conoid, but is also found together with TgCentrin-1 in the centriole. The exact association of these proteins, whether to the conoid or to the posterior polar ring, is yet to be determined.

Conoid motility into and above the posterior polar ring has been pointed out by several authors (De Souza 1974; Monteiro et al. 2001) and recorded on several occasions (Håkansson et al. 1999; Hu et al. 2006). Conoid extrusion can be artificially induced by calcium ionophore stimulation and is blocked by treatment with cytochalasin D (Mondragon and Frixione 1996). On the other hand, jasplakinolide, a membrane-permeable actin-polymerizing and filament-stabilizing drug, was unable to induce conoid extrusion (Shaw and Tilney 1999), although an amazing extension of the apical membrane containing actin filaments was observed. In situ conoid extrusion is observed during recognition and adhesion to host cells and as the parasites egress from the parasitophorous vacuole (Caldas et al. 2010). Once parasites are established in the parasitophorous vacuole inside the host cell, the conoid remains enclosed within the shell formed by the subpellicular microtubules. However, at egress, when the parasites are actively leaving the host cell, the conoid intermittently protrudes beyond the apical end of the microtubules (Hu et al. 2002; Caldas et al. 2010). Extrusion of the conoid above the polar ring is attributed by some authors (Hu et al. 2002; Morrissette 1995) to a torsion of the spiral fibers that compose it; this torsion simultaneously narrows the spiral's diameter and increases its height. However, in measurements made both in ultrathin sections and in field emission scanning electron microscopy of detergent-extracted tachyzoites of *T. gondii*, the diameter and height of the conoid were the same in the resting position as in the extruded state (unpublished data). The localization of DLC proteins around the conoid (Hu et al. 2006) is indicative of a tubulin–dynein interaction resulting in motility.

Conoid motility was demonstrated by Carey et al. (2004) to be independent from gliding motility, since ionomycin-triggered extension of the conoid was successfully inhibited by a small molecule, without interfering with gliding motility. In view of the data gathered to date, the role of calcium in triggering conoid extrusion seems undisputable. Mondragon and Frixione (1996) were first to demonstrate that calcium ionophores A23187 and ionomycin trigger conoid extrusion, as do calcium-ATPase inhibitors such as thapsigargin. On the other hand, when conoid movement was paralyzed either in the extruded or the internalized position, the ability of tachyzoites to invade cells decreased significantly, showing the relevance of motility in this process.

At this point, with the available data, microtubule-associated motor proteins seem to be more likely to be involved in conoid motility than myosin and actin filaments. However, most of the considerations made on Apicomplexa and *Toxoplasma* motility are based on an actin–myosin model.

Although actin and myosin filaments are not the focus of this review, some considerations about these structures are necessary because they may interact with the subpellicular microtubules.

9 In Trypanosomatids

Actin microfilaments were never observed in the cytoplasm of *T. cruzi*. However, cytochalasin, a drug that interferes with actin microfilaments, induces changes in the morphology of bloodstream trypomastigotes and inhibits their movement. In epimastigotes, cytochalasin causes a 48% decrease in peroxidase uptake (Bogitsh et al. 1995). Corrêa et al. (2008) demonstrated that cytochalasin B treatment leads to morphological alterations in the cytoskeletal elements associated with the cytostome–cytopharynx complex, which is responsible for transferrin uptake. Comparative genomic analysis identified a potential role for an actin–myosin system in *T. cruzi*, as this protozoan contains an actin gene as well as an expanded myosin family and a CapZ F-actin capping complex, which are not found in *T. brucei* or *Leishmania* (El-Sayed et al. 2005a, b). The authors of that study suggested that an actin–myosin system might function at the cytostome. Actin and actin-binding proteins have recently been characterized in *T. cruzi* (De Melo et al. 2008). TcActin was observed in several patch-like cytoplasmic structures, spread along the cell body of various *T. cruzi* stages, similar to actin distribution in *Leishmania* (Sahasrabudde et al. 2004). In contrast to actin in *Leishmania*, TcActin is not associated with subpellicular microtubules. Although *T. cruzi* actin is similar in structure to the actins of higher eukaryotes, homology modeling has revealed fundamental differences, predominantly in the loops responsible for oligomerization and interaction with actin-binding proteins. Consequently, actin filaments have never been detected in *T. cruzi*. Actin and actin-binding proteins (especially coronin, ADF/cofilin, profilin, formin, and myosin) have been further characterized in *Leishmania* (Reviewed in Sahasrabudde and Gupta [in press](#)).

10 In Apicomplexans

Since the initial observation of gliding motility in Apicomplexa, it has been suggested that this mechanism relies on an efficient actin–myosin-like system. However, filaments made of these proteins were never visualized in thin sections of zoites. A network of filaments with a diameter compatible with that reported for actin filaments was seen in tachyzoites of *T. gondii* using high-resolution scanning electron microscopy (Schatten et al. 2003). Subsequently, actin was isolated from subpellicular cytoskeleton extracts by binding to DNase I, and was polymerized *in vitro*. The filaments formed bound to heavy meromyosin (Parón et al. 2005). Studies using drugs that interfere with actin dynamics showed that they were able to inhibit parasite invasion in the host cells (Mondragon and Frixione 1996; Dobrowolski and Sibley 1996; Morrisette and Sibley 2002). Biochemical analysis showed the presence of actin in these organisms (Field et al. 1993; Webb et al. 1996; Dobrowolski et al. 1997a, b), probably in its globular form (G-actin). Immunofluorescence microscopy of cells incubated in the presence of antibodies

recognizing actin, but not with phalloidin, which only binds to filamentous actin, labeled the cells (Dobrowolski et al. 1997a, b). Strong evidence for the presence of actin was provided by the reaction of *T. gondii* tachyzoites when incubated in the presence of jasplakinolide, a drug that induces the polymerization and stabilization of actin filaments; under these conditions, a dramatic assembly of filaments occurred in the apical portion of the cells (Shaw and Tilney 1999). Expression of the actin gene (TgACT1) in baculovirus followed by purification of the expressed actin showed that it rapidly polymerized in vitro into filaments at a critical concentration that was significantly lower than that found for conventional actins. However, the protozoan actin filaments formed were ten times shorter and less stable than mammalian actin formed under the same experimental conditions (Sahoo et al. 2006). These observations led to the suggestion that rapid cycles of assembly and disassembly of actin in Apicomplexa govern the unusual form of gliding motility (Sahoo et al. 2006). Two excellent reviews covering basic aspects of the dynamic of actin participation in gliding motility were published recently (Schuler and Matuschewski 2006a, b).

The first evidence for the presence of myosin in Apicomplexa came from immunofluorescence microscopy using antimyosin antibodies that revealed the presence of myosin in the apical region of the cell (Schwartzman and Pfefferkorn 1983; Webb et al. 1996). Subsequently, it was shown that drugs that inhibit myosin, such as BDM and KT5926, inhibit parasite gliding motility and invasion of host cells (Dobrowolski et al. 1997a, b; Forney et al. 1998; Pinder et al. 1998). These observations were confirmed by the analysis of a conditional knockout of the small myosin motor TgMyoA (Meissner et al. 2002).

A very comprehensive model of the present knowledge on gliding mechanics was provided by Baum et al. (2006). According to this model, microneme-secreted transmembrane proteins of the TRAP family connect on the extracellular side to host cell surface components and are pushed toward the posterior end of the parasite through the aldolase–actin–MyoA machinery. The MyoA tail domain is inserted in the inner membrane complex membrane through MTIP and the whole assembly moves the parasite forward as the membrane flows to the rear end. However, the connection and functional relationship between the TRAP–aldolase–actin–MyoA–MTIP sequence and the subpellicular microtubules and the subpellicular network are not yet established or explained by a convincing model.

11 Functional Data

What are the functions played by subpellicular microtubules in trypanosomatids and in apicomplexans? It is for sure the primary scaffold for maintaining the shape. However, it also must have the plasticity to allow the interconversion between evolutive forms in Trypanosomatids and the articulation necessary for gliding motility and host cell invasion observed in Apicomplexa. Despite any apparent

similarity, subpellicular microtubules of Apicomplexa and trypanosomatids do not share many aspects in what concerns its functionality.

11.1 In Trypanosomatids

Available data point to a few functions for subpellicular microtubules in trypanosomatids. The first one is related to the maintenance of cell shape. Indeed, all the treatments that interfere with microtubules led to loss of cell asymmetry, especially in the developmental stages such as trypomastigote and epimastigote forms, with the protozoan acquiring a rounded shape. According to this view, the general shape of the cell is due to the spatial distribution of the microtubules. This distribution is probably maintained by the interaction between the microtubules and other cytoskeletal structures. This system is sensitive to signaling processes taking place during the protozoan life cycle. Indeed, transformation of an amastigote into the trypomastigote form of *T. cruzi* involves changes in the spatial distribution of the microtubules (Meyer and De Souza 1976). When amastigotes stop to divide within the host cell, the flagellar and subpellicular microtubules grow, which could bring about the elongation of the body. A second function that may be attributed to the microtubules is the maintenance of a certain rigidity of the protozoan plasma membrane, thus avoiding the assembly of endocytic vesicles and the fusion of cytoplasmic vesicles with the membrane. In addition, they preclude the direct interaction of other organelles with the plasma membrane, with the exception of the endoplasmic reticulum profiles, which are occasionally seen penetrating between the microtubules (Pimenta and De Souza 1985). Several studies have shown that endocytic and exocytic activities in trypanosomatids only take place in the flagellar pocket, whose membranes are not associated with subpellicular microtubules (Overath and Engster 2004; Field and Carrington 2004). The third possible function involves the association of the microtubules with other cell organelles such as the endoplasmic reticulum. Such connections may play a role in the maintenance of the organelles' shape as well as provide a substrate for organelle movement within the cell.

11.2 In Apicomplexans

Present evidence supports the hypothesis that subpellicular microtubules of Apicomplexa, besides their obvious role in maintaining the cell shape and polarity, participate in the gliding process by anchoring the motor proteins that provide stable support for the insertion of Tg Myo so that it can pull the actin assembly linked to the aldolase-transmembrane adhesion, resulting in the motion of the parasite (Reviewed in Baum et al. 2006). The cell body of Apicomplexa is constricted as they pass along the moving junction during invasion or along the plasma

membrane during egress (Caldas et al. 2007). This constriction is most certainly much more a consequence of the passive contraction of adjacent microtubules, as the parasite glides through a space narrower than its diameter, than mediated by microtubule-associated proteins.

The subpellicular microtubules of Apicomplexa do not restrict endo- or exocytic processes because they are not as tightly packed and linked as they are in Trypanosomatids, in order to avoid the secretion of granules; moreover, they do not extend beyond two-thirds of the length of the cell. Also, the micropore, a cytostome-like specialized structure believed to be the site of endocytosis in *T. gondii* (Nichols et al. 1994), is situated in the upper third of the cell body, indicating that the subpellicular microtubules do not restrict the internalization of nutrients at that site. The fact that the subpellicular microtubules of Apicomplexa end free at the posterior (plus) end opens up the possibility that the parasites can elongate by reducing or become shorter by increasing the distance between the subpellicular microtubules.

12 Perspectives

Subpellicular microtubules are present in Trypanosomatids and in Apicomplexans. In both the groups, microtubules are disposed side by side at regular intervals, running parallel under the plasma membrane (Trypanosomatids) and the pellicle (Apicomplexans), encaging the cytoplasmic organelles. This, along with the fact that α/β -tubulin dimers are their main constituent, is the main and almost the only similarity that both types of microtubules share. The regular bridges that link the subpellicular microtubules of trypanosomatids turn them into rigid nutshells unable to bend or constrict the cell body, as is observed in *Toxoplasma*, *Plasmodium* and other Apicomplexa. However, because microtubules in trypanosomatids form helical sheets, they can swell or shrink under osmotic stress, causing the cells to become shorter and spherical or corkscrew-shaped, as observed in *Phytomonas staheli* (Attias et al. 1987). The subpellicular microtubules of Apicomplexa, on the other hand, react to variations in the osmotic pressure of the environment by spreading like the ribs of an umbrella.

At this point, although a lot of information both on the ultrastructure and on the proteomics of subpellicular microtubules has been gathered, the correlation between these data is still missing. Elucidating this correlation will be necessary for the identification of the proteins that link subpellicular microtubules to each other, to the plasma membrane and to cytoplasmic organelles such as mitochondria and the endoplasmic reticulum. The identification of a microtubule-organizing center in Trypanosomatids certainly would be helpful in explaining how they change their shape during their life cycle.

As for Apicomplexa, the challenge is not smaller and several points remain obscure or disregarded. Among them is the question of the actual role played by conoid motion in gliding and invasion. Parasites lacking conoids, such as

Plasmodium, are as competent in invading cells as *Toxoplasma* and *Eimeria*, which have conoids. Moreover, the regulation and mechanics of conoid motion and conoid interaction with motor proteins are not yet understood.

It is known that micronemes and rhoptries secrete their contents at the apical portion of the parasite, but it is not known whether conoid positioning above or below the polar ring is necessary for this secretion to occur. The same applies to the central pair of microtubules: its role and relation to other components of the cytoskeleton and secretory organelles remains obscure. Another point that needs clarification is how adhesion proteins secreted by micronemes are incorporated into the plasma membrane to bridge extracellular molecules to intracellular actin–myosin and the related protein machinery that is involved in gliding.

The subpellicular network is another enigmatic structure; very little is known about its exact structure, location, or function.

The anchoring of the actin–myosin motor to subpellicular microtubules, mediated by the inner pellicle, is another point about which there is much left to elucidate, including the role of these microtubules in cell shape and motility. All these questions are exciting and intriguing puzzles that should receive attention and be cleared out in the years to come through the use of molecular, biochemical, and ultrastructural techniques.

Acknowledgments The work carried out at the authors' laboratory was supported by CNPq/MCT, FINEP, CAPES, DECIT, and FAPERJ.

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Structures and Organelles in Pathogenic Protists

de Souza, W. (Ed.)

2010, XII, 324 p. 78 illus., 19 illus. in color., Hardcover

ISBN: 978-3-642-12862-2