IMMUNOCYTOCHEMICAL DIFFERENTIATION OF MICROTUBULES IN THE CYTOSKELETON OF GIARDIA LAMBLIA USING MONOCLONAL ANTIBODIES TO α-TUBULIN AND POLYCLONAL ANTIBODIES TO ASSOCIATED LOW MOLECULAR WEIGHT PROTEINS

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SUMMARY

In interphase trophozoites of Giardia lamblia, separate populations of microtubules constitute the four parts of the mastigont apparatus: flagella, ventral disc, funis and median body.

Antigenic differences between the tubules have been investigated by light and electron immunocytochemistry after labelling with two monoclonal antibodies to α-tubulin (YL 1/2 and YOL 1/34 clones), and with polyclonal antibodies to Giardia tubule-associated proteins. Both anti-tubulins stained all tubules after isolated structures were fixed in formaldehyde, but different patterns of reactivity were shown by unfixed tubules. YL 1/2 antibodies labelled flagellar axonemes and basal bodies, funis and median body tubules. Disc microtubules were mostly unlabelled, but the antibody bound strongly to the outer edge of the disc where the ends of tubules are embedded. YOL 1/34 antibodies stained disc tubules uniformly, and cross-reacted with the median body but not with tubules of axonemes, basal bodies or funis.

Antibodies to giardins 14A and 14B (~30,000 M₅ filament-forming proteins) localized these proteins in the microribbons attached to disc microtubules. The median body was also labelled by anti-giardins, indicating an ontogenetic relationship between this organelle and the ventral disc.

A second set of ~30,000 M₅ proteins with no immunoreactivity to anti-giardin was found in flagella purified without removing flagellar membranes. These polypeptides were Triton-soluble and therefore probably originated from an extra-axonemal site. A rabbit antiserum to the labile flagellar proteins specifically stained the two ventral flagella, but not the other six flagella on this cell.

INTRODUCTION

It has long been recognized that separate functions are served in the cytoplasm by subpopulations of microtubules that can be distinguished by subtle differences in morphology (Behnke & Forer, 1967; Eichenlaub-Ritter & Tucker, 1984) or stability (Brown & Bouck, 1973). At a chemical level, microtubules from different sites may

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have characteristic patterns of tubulin isotypes, for example in the cytoskeleton and flagella of the algae *Polytomella* and *Chlamydomonas* (McKeithan, Lefebvre, Silflow & Rosenbaum, 1983), or differences in microtubule-associated proteins (MAPs) (Huber & Matus, 1984).

Trophozoites of the zooflagellate *Giardia* have differentiated sets of microtubules, which make up four different parts of the mastigont apparatus: the ventral disc cytoskeleton, the funis, the median body and the flagella (Kulda & Nohynkova, 1978; and see Fig. 1). The different microtubules are located precisely within these structures (Holberton, 1973; Brugerolle, 1975). Recently, tubulin determinants have been localized separately in the disc cytoskeleton and flagella using three different monoclonal anti-tubulins prepared against *Giardia lamblia* antigens (Torian, Barnes, Stephens & Tibbs, 1984). It is known also that there are at least five isoelectric variants of *Giardia* tubulin (Crossley & Holberton, 1983a).

In the disc, microtubules have certain protofilaments bound to large microribbons and possess sets of paired arms that link to the ventral membrane (Holberton, 1973; Holberton & Ward, 1981). Isolation of the cytoskeleton has shown nearly 20 polypeptides copurifying with tubulin. Prominent amongst these are the giardins, a family of components with polypeptide chain $M_r$ values close to 30,000. The major giardin species reassemble in vitro into ribbon-like structures (Crossley & Holberton, 1983a, 1985), and are probably structural proteins of the microribbons. However, by isoelectric focusing (IEF) the giardins are found to be a heterogeneous group and may include chemically distinct molecules having interactions with microtubules at other sites in the cytoskeleton.

In the present study, monoclonal antibodies to yeast spindle tubulin (Kilmartin, Wright & Milstein, 1982) and polyclonal antibodies to giardins have been used as direct probes of chemical differences amongst *Giardia* microtubules.

**MATERIALS AND METHODS**

**Preparation of cytoskeletons and extraction of proteins**

An axenic strain of *G. lamblia* was grown in Diamond's TPS-1 medium (Visvesvera, 1980) in 2.5-l reagent bottles. Cells were harvested after 4 days of culture by cooling to 4°C and centrifuging at 4000 $g$ for 10 min. Cytoskeletons were prepared by washing cells three times in 200 ml of cold 10 mM-MES, 150 mM-KCl, 2 mM-ATP, 2 mM-EDTA, 1 mM-dithiothreitol (DTT), pH 6.7, and extracting in the same buffer with 0.5 % (v/v) Triton X-100 (Crossley & Holberton, 1985).

To extract giardins, washed cytoskeletons were dialysed for 90 h at 4°C against 10 mM-HEPES, 5 mM-EDTA, 0.05 mM-DTT, pH 8.6–8.7. After pelleting insoluble residues at 200,000 $g$ for 1 h, giardin was repolymerized from the supernatant fraction by dialysis against isotonic KCl at 4°C for 24–48 h (Crossley & Holberton, 1985).

The protein concentration in experimental samples was determined by binding Coomassie Brilliant Blue (Bradford, 1976). The absorbance at 595 nm was calibrated from bovine serum albumin (BSA) standards.

**Sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE)**

Electrophoresis was carried out in a continuous buffer system using gels prepared by the method of Stephens (1975). For analytical electrophoresis, samples in 2% SDS were run on vertical 170 mm long, 5% to 15% or 5% to 20% polyacrylamide gradient gels cast to a thickness of
0·8 mm. Runs were for 2–3 h at a constant current of 20 mA in a water-cooled tank at 20°C. Gels were stained overnight in 0·2 % (w/v) PAGE Blue 83 (BDH) then destained as before (Crossley & Holberton, 1985).

Preparative electrophoresis was on 6 mm thick vertical slabs cast as a 150 mm separating gel of 10 % polyacrylamide, with a 60 mm stacking gel of 3 % polyacrylamide. A long sample well cast around a perspex former measuring 115 mm x 20 mm x 6 mm was loaded with 5 mg of cytoskeleton proteins in 1 ml of SDS sample buffer. Gels were run at 50 mA constant current. Strips from the edges of the gel were stained for 10 min in Bradford's reagent (Bradford, 1976), and used to locate the positions of bands. The horizontal strip of gel with the separated giardins was homogenized in 5 ml of running buffer and the slurry was electroeluted into a dialysis bag (Stephens, 1975). The eluted giardin sample suspended in a small volume of buffer was stored at −20°C.

Preparation of antisera
Rabbits were immunized with 50 μg of giardin mixed with Freund's complete adjuvant, injecting at multiple sites. Four weeks later, 25 μg of protein in Freund's incomplete adjuvant were injected at a single site. At intervals, immune serum was tested for cross-reaction with assembly-purified giardin (50 μg ml⁻¹) on Ouchterlony plates. At peak titres, rabbits were bled out and the immunoglobulin G (IgG) was precipitated in 50 % ammonium sulphate and fractionated on DEAE-Trisacryl (Kilmartin et al., 1982).

The rat monoclonal antibodies YOL 1/34 and YL 1/2 (to yeast spindle tubulin) were gifts from Dr J. V. Kilmartin (Medical Research Council, Cambridge).

Protein blotting
Blots were prepared on nitrocellulose sheet (0·22 μm pore size, Schleicher and Schüll) by electrophoretic transfer from gradient gels in 20 % (v/v) methanol, 25 mM-Tris-glycine, pH8·3 (Towbin, Staehelin & Gordon, 1979). Blots were quenched for 1 h at 37°C in 3 % BSA in 10 mM-Tris, 154 mM-NaCl, pH7·4. To label bands, blots were incubated for 2·5 h in immune rabbit IgG (0·8 mg ml⁻¹) diluted 1:100 in Tris-saline with 1 % goat serum. After rinsing in three changes of Tris-saline, the blots were developed overnight in gold-labelled goat anti-rabbit IgG (GARG 5, Janssen Pharmaceutica), diluted 1:200.

Immunofluorescence
Cells were prepared in 0·25 M-sucrose on poly-L-lysine(0·1 %)-coated glass slides. After rinsing in phosphate-buffered saline (PBS), attached cells were demembranated by irrigating with TEDAMP buffer (without ATP) + 0·5 % Triton X-100 (Crossley & Holberton, 1983a). Slides were washed with PBS or placed directly in 3·5 % formaldehyde in PBS for 15 min. Both fixed and unfixed samples were washed in PBS with 2 % normal goat serum for 30 min before immunolabelling at 37°C for 2 h. Primary antibodies were used at dilutions of 1:100 in PBS; polyclonal antibodies were used with 1 % normal goat serum. After washing in PBS, slides were incubated in second antibodies at 37°C for 1 h. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat IgG (Sigma) with 1 % normal rabbit serum, and FITC-goat anti-rabbit IgG (Miles) with 1 % normal goat serum, were used as second antibodies for the monoclonal and polyclonal antibodies, respectively.

Alternatively, air-dried trophozoites on clean coverslips were fixed in 3·5 % formaldehyde, then permeabilized in cold acetone and stained with a double layer of antibodies (Lazarides, 1976), using 10 mM-Tris, 154 mM-NaCl buffer (De Mey et al., 1981).

Preparations in the fluorescence promotor 'Citifluor' (City University, London) were viewed with a Leitz Ortholux microscope equipped with epifluorescence optics.

Electron microscope (EM) immunocytochemistry
Isolated cytoskeletons were processed as described by De Mey et al. (1981), and fixed in 2·5 % glutaraldehyde after incubation with the final antibody. Goat anti-rabbit IgGs labelled with 10 nm or 20 nm gold particles (GARG 10 or GARG 20, Janssen Pharmaceutica) were used to detect the
first antibodies. For the rat monoclonal antibodies to tubulin, rabbit anti-rat IgG (Miles) was used to bridge to GARG 20. After fixing, the cytoskeletons were rinsed in buffer then postfixed in 1% osmium tetroxide, before dehydrating and embedding as before (Crossley & Holberton, 1983a).

Repolymerized filaments were labelled with anti-giardins on 0.1% poly-L-lysine films on Formvar-coated copper grids. Grids were washed in 20 mM-Tris at pH 8.2 with 154 mM-NaCl, 0.02% sodium azide, containing in turn: 10% BSA; 1% BSA; 5% normal goat serum; 1% normal goat serum + 5% immune rabbit IgG (first incubation); three washes of 1% normal goat serum. All steps were for 10 min each at 20°C. Grids were then incubated for 30 min at 37°C in buffer with GARG 10 in 1% BSA (second incubation). Three washes in Tris-buffered saline with 1% BSA were followed by two washes in PBS, before fixing in 1% glutaraldehyde in PBS. Grids were rinsed in distilled water before staining in 0.5% uranyl acetate at pH 4.7. Controls were prepared with the first incubation in goat serum either without immune rabbit IgG, or with pre-immune rabbit serum diluted 1:20.

Stained sections and whole mounts of cytoskeletons or filaments were viewed and photographed at an accelerating voltage of 80 kV in a JEOL 100C electron microscope.

RESULTS

**Differentiation of Giardia microtubules**

Fig. 1 shows the microtubules that constitute the flagellar axonemes and three different parts of the cytoskeleton in *G. lamblia*. When prepared carefully, the cytoskeleton can be displayed in its entirety without displacing microtubules from the positions they occupy *in situ*.

Two microtubular structures of the mastigont, the disc and the funis, are associated with the basal bodies of the flagellar axonemes. The funis is a framework of microtubules that originate near the basal bodies and run above and below the caudal axonemes into the tail as a posterior cytoskeleton. The median body is a separate and

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**Fig. 1.** Four discrete sets (1-4) of microtubules in the cytoskeleton of *G. lamblia*, as seen from above. The cytoskeleton is displayed by attaching trophozoites to a polylysine-coated grid and washing away the plasma membrane and cytosol with 0.5% Triton medium. Frequently, as here, the two nuclei (n) remain attached to the basal body complex. The large disc cytoskeleton (1) is a sheet of microtubules bearing microribbons (seen in section in Figs 11-13), which are concentrically wound in a right-handed spiral of between 1.25 and 1.75 turns to give a region of overlap on the anterior right side. At the proximal end of this sheet is a laminated plaque or collar, here banded with three light and two dark lines, to which is anchored a group of four basal bodies (a, p, v, c) lying to the left of the midline. On the right a similar set of basal bodies is associated with a second, smaller, banded plaque alongside the first. The second plaque gives rise to a homologue of the disc, a truncated sheet of tubules that crosses to the left alongside the anterior axoneme. The second group of microtubules comprises the axonemes of the four pairs of flagella: anterior (2a), caudal (2c), posteriolateral (2p) and ventral (2v), arising from the central cluster of basal bodies. The third set makes up the funis (3). This structure is a girdle of parallel singlet tubules that partly encloses the caudal axonemes from the basal bodies to the posterior edge of the disc. Beyond the disc, the tubules fan out above and below the axoneme bundle to provide a set of lateral ribs reaching into the posterior cytoplasm. The dorsal group arises from the region of the left group of basal bodies, but crosses to the right side of the organism; similarly, the ventral group arises near basal bodies on the right, but the tubules terminate beneath the membrane on the left side. The median body (4) is loosely attached to the funis and caudal axonemes, and contains the fourth group of tubules, which are all more or less transversely oriented (in *G. lamblia*) in one or more compact bundles (seen in section in Fig. 14). Negatively stained in 0.5% sodium tungstate, pH 7.2. Bar, 1 μm.
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2a

a

v

c

p

n

n

3

4

2p

2c

2v
compact bundle of microtubules lying across the top of the funis, just behind the ventral disc.

It is difficult to maintain the cytoskeleton in this intact form in bulk preparations. When cytoskeletons are isolated by suspending cells in Triton media without first attaching to a surface, the median bodies and tubules of the funis are normally lost into the supernatant (Holberton & Ward, 1981).

Anti-tubulin recognition of different microtubules

In fixed permeabilized cells or fixed isolated cytoskeletons, the different microtubules stained uniformly with either of the monoclonal antitubulins YL 1/2 or YOL 1/34 (Fig. 2). At the concentration used, the fluorescence given by YOL 1/34 was much brighter than that given by YL 1/2. The staining of the median bodies was especially intense, presumably because of the tight packing of numerous microtubules in this structure.

To show specific staining of different microtubules, cytoskeletons were prepared on polylysine films and cross-reacted without fixing. After labelling with FITC-conjugated second antibodies, YL 1/2 gave a distinctive pattern of fluorescence, which faded quickly. The disc, despite its large number of microtubules, did not generally stain with YL 1/2 except for a bright rim of fluorescence around its edge.
Giardia microtubule immunochemistry

Other structures of the unfixed cytoskeleton were labelled clearly by YL 1/2. These included the median body and the basal bodies (Fig. 3D). A diffuse wedge of fluorescence between the basal bodies and median body region indicated that there was some cross-reaction with tubules of the funis. The flagellar axonemes were also labelled by YL 1/2 but in a patchy non-uniform manner. Flagellar fluorescence faded rapidly.

The labelling of unfixed cytoskeletons by YOL 1/34 was very different. Bright fluorescence was confined to the disc and median body, and was persistent; in different preparations there was either very weak staining or no reaction at all with tubules of the flagella or funis (Fig. 4). The disc plate stained uniformly and showed clearly the shape of the organelle, including the region of overlap (Fig. 4B).

The specific labelling of unfixed disc tubules was examined also at the ultrastructural level using gold-tagged final antibodies.

Funis tubules and median bodies were not found in pellets of cytoskeletons prepared for sectioning. The labelling of profiles of discs confirmed the fluorescence studies. YL 1/2 antibody failed in general to decorate disc structures, but clusters of gold particles were found in a few cases around the outermost microtubules (Fig. 11). It was not obvious from the local ultrastructure why tubule profiles at these sites should be distinguished from the similar profiles elsewhere in the disc. Disc tubules originate from either the left laminated collar or the inner edge of the disc, and spiral out to the outer rim (Holberton & Ward, 1981). Labelling close to the rim thus represents a specific cross-reaction with the ends of the tubules that are unrecognised in the body of the disc.

Axonemes were mostly unlabelled by YL 1/2, but very occasionally groups of labelled antibodies were found adhering to one or two doublet microtubules (Fig. 11c).

Immunogold-labelled YOL 1/34 anti-tubulin reacted specifically with disc microtubules and not at all with axonemal tubules. Fig. 12 shows a line of gold particles decorating the disc tubules where YOL 1/34 has bound to their exposed (ventral) surfaces. On the dorsal side, the microribbons attached to the tubules obstruct the access of antibodies to tubule protofilaments.

The microribbons themselves were not labelled in these sections by monoclonal antibodies of either type.

Characterization of polyclonal antibodies

The sera from rabbits injected with cytoskeleton proteins were assayed for antibody titre on Ouchterlony double-diffusion agar plates. Control assays using pre-immune sera showed no detectable antibodies to giardin before injection. Specific antibodies were induced in all rabbits injected with pure giardin at 50 µg ml⁻¹. The immune sera gave single precipitin lines at dilutions up to 16-fold when diffused against giardin. There was no cross-reaction between anti-giardin and tubulin.

The specific polypeptides recognized by anti-giardin antibodies were identified on protein blots prepared from gradient gels. Staining of the gels after transfer of
the proteins showed that most cytoskeleton polypeptides had been transferred completely with the exception of some residual minor bands of high \( M_r (>90000) \).

Two antisera (designated R4 and R5 antibodies) were characterized in this way and gave similar results. Fig. 5 shows that only bands in the giardin region of the cytoskeleton protein fractions were labelled. The antibodies principally recognized giardins of bands 14A and 14B (numbered according to the scheme introduced by Crossley & Holberton, 1983a), although a faint cross-reaction with a faster component in the region of band 15 was detectable. It is the giardins that are soluble in solutions of low ionic strength, of bands 14A, 14B and 15, that form fine 2·5 nm filaments \textit{in vitro} (Crossley & Holberton, 1985). The non-reacting \(~30000 M_r\) bands in cytoskeleton polypeptide preparations (band 13, Fig. 5; and see Fig. 9) may be related to similar polypeptides in \textit{Giardia} flagella, although the flagellar components tend to be labile to Triton extraction (see below).

Blots were used also to check that the monoclonal antibodies YOL 1/34 and YL 1/2 did not cross-react with other \textit{Giardia} cytoskeletal proteins sharing determinants with tubulin. Using FITC-labelled rabbit anti-rat second antibodies to visualize the monoclonal antibodies, fluorescence was detected only at the \( \alpha \)-tubulin level.

\textit{Decoration of in vitro polymerized giardin filaments}

Bundles of giardin filaments were repolymerized (Fig. 6) from a cytoskeleton protein fraction soluble in low salt buffer (Fig. 5A, track 1) by dialysing this fraction back to isotonic KCl (Crossley & Holberton, 1985). Analysing the sedimented material showed that giardin chains 14 and 15 accounted for most of the stained material on SDS/polyacrylamide gels (Fig. 5A, track 2). To confirm that these filaments were formed from giardin, samples were collected on coated grids and processed for immunoelectron microscopy by incubating with R5 antibodies. The immunostained filament bundles seen in the EM were heavily coated with gold particles (Fig. 6B). The double antibody layer obscured fine details, but longitudinal filaments could still be resolved in some decorated bundles, as could some of the transverse cross-striations at 15-nm intervals, which represent a repeat fundamental
to the packing of giardin into polymer structures (Crossley & Holberton, 1983b, 1985). Some grids were incubated in goat serum or goat serum with pre-immune rabbit serum. In these controls there was no visible gold labelling of filaments.

**Localization of disc giardins (band 14 and 15 polypeptides)**

Isolated cytoskeletons and permeabilized* Giardia* trophozoites were immunostained for the presence of giardins, using the polyclonal R5 antibodies. The distribution of the FITC-labelled second antibodies was localized strongly in the disc region of the cell and in the median bodies, which were brightly labelled (Fig. 7). Isolated discs fluoresced with a clarity comparable to the staining obtained with the monoclonal anti-tubulin YOL 1/34. There was no labelling of the exposed tubules of the demembranated axonemes and the associated funis, nor of the more intact flagella of permeabilized cells. This result distinguishes R5 anti-giardin determinants from the labelling obtained with an antiserum to giardin-sized polypeptides derived from flagella (see below).

When discs were examined in the electron microscope, colloidal gold labelling was restricted to the microribbons, the region around disc microtubules being free from label. Transverse sections through discs showed that the antibodies attached strongly to the free (dorsal) edges of microribbons, which were heavily decorated (Fig. 13). Few of the gold particles were found at the faces of ribbons, even where these structures had become sufficiently separated to permit access of antibodies to these sites.

**Flagellar ~30 000 M<sub>r</sub> components**

A set of ~30 000 M<sub>r</sub> components is known to be associated with* Giardia* flagella (Clark, 1985). Flagella can be prepared from* Giardia* by mechanical shock, followed by centrifugation to remove cell bodies. The crude flagellar sample is then

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*Fig. 5. SDS/PAGE of giardins and immunocharacterization of R5 antibodies. A. 5% to 20% polyacrylamide gel of fractions from the assembly purification of giardins (first cycle). Stained with PAGE Blue 83. Track 1: supernatant of soluble proteins extracted from cytoskeletons at low ionic strength in HEPES/EDTA buffer, pH 8-5. The sample contains tubulin (α and β), and giardin chains 13–15. Track 2: composition of filaments precipitated from HEPES/EDTA buffer by dialysis against 150 mM-KCl (Crossley & Holberton, 1985). B. Replica blot onto nitrocellulose of proteins from the gel of A, immunostained with R5 rabbit polyclonal antibodies followed by gold-labelled goat anti-rabbit IgG (GARG 5). Only components from the giardin region are recognized by R5 antiserum. Two heavily labelled bands correspond to chains 14A and 14B.

*Fig. 6. Giardin filament bundles prepared by repolymerizing band 14 and 15 polypeptides at elevated ionic strength. Negatively stained in 0·5% uranyl acetate, pH 4-7. A. Unfixed paracrystal of giardin showing characteristic banding pattern. The interval between dense bands is 15 nm. Bar, 50 nm. B. Filament bundle from the same experiment at lower magnification, reacted in situ on the grid with R5 anti-giardins (first antibody) at 1:20 dilution + goat serum, followed by 10 nm gold–goat anti-rabbit IgG (second antibody). Filaments and tactoids were heavily encrusted with gold-labelled antibodies. Some of the dissociated material in the background also labels with anti-giardin. Bar, 100 nm.*
Fig. 7. Indirect immunofluorescence of unfixed cytoskeletons on polylysine stained with R5 polyclonal anti-giardins, followed by FITC-goat anti-rabbit IgG. There was no fluorescence from controls in which anti-giardins were replaced by preimmune rabbit serum. A,C. Phase-contrast micrographs of a group of extracted cytoskeletons, and a single cytoskeleton with a median body. Bars, 10 μm. B,D. Corresponding fluorescence micrographs showing bright anti-giardin staining of discs and median body.

fractionated by centrifuging on a self-generating Percoll gradient, on which the organelles occupy a broad density zone between 1.076 and 1.111 g ml⁻¹. In this pure fraction, flagella are still bounded by the surface membrane and their polypeptide composition includes a number of bands that are labile to demembranation. These components incude bands that broadly comigrate with the giardin bands of Triton-insoluble cytoskeletons (Fig. 8).

The immune cross-reactions of flagellar proteins and cytoskeleton giardins were compared on protein blots from polyacrylamide gels run with the corresponding samples. Fig. 9 shows that none of the flagellar 30000 Mᵦ components was recognized by R5 anti-giardins, and they were therefore confirmed as species distinct from the giardins obtained from the disc cytoskeleton. The lability of the flagellar bands suggests that the proteins are not integral to the axonemes per se, which can be reactivated with ATP and therefore remain functional after Triton extraction (Holberton & Ward, 1981).

One possibility is that these components derive from the paraxial rods, found only in the ventral flagella, which, though linked structurally to doublet tubules (Holberton, 1973), are removed with the flagellar membranes during Triton treatment. To examine this possibility, a rabbit antiserum was raised to the flagellar 30000 Mᵦ components after the appropriate bands were cut from several gels,
pooled, and electroeluted to yield approximately 150\,\mu g of denatured protein, which was used as the immunogen. Permeabilized cells treated with the antiserum and stained with FITC-labelled goat anti-rabbit IgG gave the result shown in Fig. 10B. The antiserum specifically labelled the two ventral flagella, which fluoresced brightly. Because the basal bodies are found above the front of the disc, the axonemes of the posteriorly directed flagella run within the cytoplasm for some distance before emerging behind the disc as flagella. In the immunostained cells only the free flagella were labelled; the intracytoplasmic parts of the two ventral axonemes did not bind the antibodies. It is likely, therefore, that the 30\,000\,M_r polypeptides are associated either with the flagellar membrane or with the paraxial rods beneath the membrane that run alongside the axoneme from its point of emergence to the flagellar tip.

Fig. 8. SDS/PAGE on a 5\% to 15\% polyacrylamide gradient gel, comparing the proteins of purified flagella from \textit{G. lamblia} (track 2), and axonemes of the same preparation after removing membranes by Triton extraction (track 1). In whole flagella the major components are tubulin (\(\alpha\) and \(\beta\)) and, close to 30\,000\,M_r (calibrated), a set of four chains that are largely Triton-labile. Stained in PAGE Blue 83.

Fig. 9. Comparison of disc cytoskeleton giardins and flagellar components of similar mobility. A. SDS/PAGE of flagellar bands (track 1) and giardin chains 13–15 in Triton-extracted discs (track 2), stained with PAGE Blue 83. The major flagellar chain has a mobility slightly ahead of giardin 14A. B. Immunostaining of the same proteins after electrophoretic transfer onto nitrocellulose. R5 polyclonal antiserum followed by gold-labelled goat anti-rabbit IgG (GARG S). Cytoskeleton giardins are stained by R5, but there is no comparable cross-reaction with flagellar components.

Fig. 10. Indirect immunofluorescence of formaldehyde-fixed, acetone-permeabilized trophozoites. The primary antibody was a rabbit antiserum raised to \(\sim 30\,000\,M_r\) bands excised from SDS/PAGE gels of a pure flagellar fraction. The rabbit antibody stained the same flagellar bands in immunoblot tests. Antibody binding to trophozoites visualized with FITC-goat anti-rabbit IgG. A. Phase-contrast micrograph of a single trophozoite with extended anterior (a), posteriolateral (p), and caudal (c) flagella. The ventral flagella (v) are coiled close to the body surface. Bar, 5\,\mu m. B. Corresponding fluorescence pattern showing diffuse staining of the cell body, and bright fluorescence from the two ventral flagella. The other flagella are completely unlabelled.

Figs 11–13. Electron-immunocytochemistry of isolated cytoskeletons. Cytoskeletons were pelleted, fixed and embedded after treating with antibodies and washing.

Fig. 11. Cytoskeletons reacted with YL 1/2 monoclonal anti-tubulin, followed by rabbit anti-rat IgG, and gold-labelled goat anti-rabbit IgG (GARG 20). A. Low-power view of four sectioned discs. Few gold particles are attached to the microtubules in these structures, except for the very edge of one disc (arrow). Bar, 500\,nm. B. A similar disc profile at higher magnification showing a cluster of 20\,nm gold particles at the edge of the disc. Bar, 200\,nm. C. Cross-section of a pair of axonemes. Gold particles appear to bind to two of the doublet tubules in one axoneme (arrow); axoneme profiles were either unlabelled or labelled in this limited way by YL 1/2. Bar, 200\,nm.

Fig. 12. Labelling of disc microtubules by YOL 1/34 monoclonal anti-tubulin. The line of gold particles alongside the tubule profiles is typical of the labelling obtained with this antibody. Axonemes in the same sections were completely unlabelled. Bar, 200\,nm.

Fig. 13. Section through three discs (one transversely sectioned and two obliquely sectioned) showing the result from immunogold labelling by polyclonal R5 anti-giardins and gold-labelled goat anti-rabbit IgG (GARG 10). Heavy labelling along the top edge of microribbons is typical. There are a few gold particles between ribbons, but note that the ribbon faces are mainly unlabelled. Bar, 200\,nm.
Figs 8–10. For legends see p. 245
Figs 11–13. For legends see p. 245
DISCUSSION

Tubulin determinants

The antibodies of the hybridoma clones YL 1/2 and YOL 1/34 have been selected for their ability to bind to external domains on fixed yeast microtubules (Kilmartin et al. 1982). Binding the same antibodies to the equivalent sites on brain tubulin does not interfere with polymerization–depolymerization processes, nor does it impede association of high molecular weight microtubule-associated proteins or tau proteins with brain microtubules (Wehland, Willingham & Sandoval, 1983). Both antibodies bind specifically to the denatured chains of α-tubulin subunits from rat and bovine brain (Wehland et al. 1983), rat cerebellum cytosol (Cumming, Burgoyne & Lytton, 1984), pig brain and simple eukaryotes like Physarum (Chang, Kilmartin & Dove, 1981).

The determinant recognized by YL 1/2 has now been identified as the carboxy terminal sequence of α-tubulin chains, when this terminal is in the tyrosylated form (Wehland, Schroder & Weber, 1984). Detyrosylation in situ of fixed tubules in sections of rat cerebellar tissue, using carboxypeptidase A, abolishes subsequent immunostaining by YL 1/2 antibodies, but not by YOL 1/34 (Cumming et al. 1984). There is also evidence that the different reactivities of YL 1/2 and YOL 1/34 antibodies distinguish some of the rat brain α-chain isotypes separated by IEF. Other results from using these two antibodies have shown that sets of tubules with different labelling patterns can be identified in fixed cells, for example during postnatal differentiation of rat cerebellum (Cumming et al. 1984), and in trypanosomes (Gallo & Anderton, 1983; Cumming & Williams, 1984).

In the results from immunostaining isolated Giardia cytoskeletons, the ability of the two monoclonal IgGs to discriminate between tubules at different sites is shown clearly only on unfixed microtubules. Once tubules have been fixed in formaldehyde both antibodies label all parts of the cytoskeleton. Therefore, the absence of a reaction between YL 1/2 and unfixed discs, and between YOL 1/34 and unfixed axonemes, basal bodies and funis tubules, is not, presumably, due to the segregation of alternative α-tubulin isotypes in different parts of the cytoskeleton. This leaves the possibility that determinants recognized on some microtubules after fixation are not available for antibody binding when the same tubules are not fixed. Unfixed proteins may be susceptible to modifications directly affecting the subunit antigenicity (for example, the loss of carboxy terminal tyrosine from the YL 1/2 epitope). Alternatively, fixing might distort polypeptide conformation or the bonding arrangement between subunits and associated proteins to expose determinants masked in the native structure of some tubules. The latter effect has been suggested as the reason why antibodies to tektins (flagellar tubule proteins) stain purified sea-urchin axonemes fixed in a denaturing buffer, but not when MgCl₂ and EDTA are included to preserve tubule structure (Linck, Amos & Amos, 1985).

There have been few studies of the binding of YL 1/2 and YOL 1/34 antibodies to unfixed microtubules. In one study, these two antibodies had different effects on the cytoskeleton of live Swiss 3T3 fibroblasts when microinjected into the cytoplasm.
YL 1/2 interrupted microtubule-associated functions (intracellular transport, translocation), eventually inducing the formation of massive tubule bundles (Wehland & Willingham, 1983). YOL 1/34 had none of these effects; by immunofluorescence it was found to be distributed diffusely in the cytoplasm and was not associated with any part of the tubule network. However, both monoclonal antibodies label the microtubule cytoskeleton in fixed 3T3 cells (Wehland et al. 1983).

In the present work we found that unfixed axonemal microtubules were decorated only by YL 1/2 and not by YOL 1/34 antibodies, and that YL 1/2 binding was variable and intermittent along tubules. This result can be compared with observations from unfixed *Psammechinus miliaris* sperm tails (Lincke et al. 1985), in which it was found that YL 1/2 attached to dissociated singlet A-tubules or to protofilament ribbons, but not to intact doublets where the determinants were masked by other components. Similarly, our result could be explained by assuming that doublet structure had broken down in those regions of the *Giardia* axonemes reacting with the antibody.

**Localization of giardins**

The rabbit antisera to purified giardin cross-reacted specifically with chains 14A and 14B, enabling us to localize these polypeptides in the cytoskeleton. Earlier work established that it is these chains in particular that have structural characteristics leading to the self-assembly of ribbon structures in vitro (Crossley & Holberton, 1983b, 1985). We have suggested that in vivo the microribbon is a sandwich of protein layers in which the central layer of giardin is attached to a disc microtubule, and provides a framework to which the outer protein layers are bound. The localization of giardins 14A and 14B in cytoskeletons corresponds to the precepts of this model. Anti-giardins were attached to a microribbon at its free edge, which is the only point at which we would expect the inner core to be exposed. A significant result is that despite the high content of giardins in discs, the two faces of a microribbon were unrecognized by polyclonal anti-giardins, confirming that these surfaces are made up of some additional component.

We have also identified ~30,000 Mr polypeptides from flagella that are antigenically different from the ribbon-forming giardins. On SDS/polyacrylamide gels there are at least three close-spaced flagellar bands, of which the middle and most prominent polypeptide is co-migratory with band 14 giardins from disc cytoskeletons. When purifying giardins 14 and 15 by gel filtration, there is a late elution of a second set of ~30,000 Mr polypeptides, which appear to correspond (in terms of mobility on SDS/polyacrylamide gels) to the flagellar components. From the elution position, these chains are in compact particles, and therefore are physically as well as chemically unlike the filamentous giardins.

**Microribbon tubulin?**

The electron-immunochemical results show that the faces of native microribbons are unreactive to either monoclonal anti-tubulins or polyclonal anti-giardins. These layers consist of regular lattices of stained features the size of tubulin monomers. For
Fig. 14. Cross-section through a median body in a fixed trophozoite of *G. muris*. Microtubules mostly have the same orientation and appear to be embedded in an unstructured matrix of moderate electron density. Many of the tubules have a short dense projection or spur on one side (arrows). In *G. muris* tubules in the median body are oriented longitudinally and parallel with tubules in the funis (f) and caudal axonemes (c). Bar, 100 nm.

This reason, it was proposed earlier that microribbons were sheets of tubulin bound to a central giardin lattice (Holberton, 1981). The two anti-tubulins used in the present study failed to decorate these layers, so their chemical identity remains unconfirmed. However, YL 1/2 and YOL 1/34 antibodies have been screened for a positive immunofluorescence against polymerized tubules (Kilmartin et al. 1982). Thus the epitopes of these antibodies are regions of the α-tubulin molecule facing outward after normal assembly of tubules. Since the giardin core of the ribbon is attached to protofilaments of the disc microtubule, it is to be expected that some giardin-binding domains will be found on the outside surfaces of microtubules. It seems quite probable that tubulin at the faces of ribbons would be attached to the giardin
framework through the same sites. In other words, the orientation of microribbon tubulin might be such that the face presented to the outside is the surface normally on the inside of a microtubule. We would not expect these surfaces to be recognized by YL 1/2 and YOL 1/34 antibodies.

The median body

A significant result is that the median body contains determinants for both monoclonal anti-tubulins and for anti-giardins. The localization of giardin in this organelle requires further investigation by immunolabelling at the electron-microscope level. However, it is likely that median body giardin is associated directly with microtubules, and there is additional evidence to support this. In *G. muris*, at certain stages of the life cycle, some median body microtubules are attached to fully developed microribbon-like structures (Brugerolle, 1975). More usually, sections of the median body in either *G. muris* or *G. lamblia* appear as in Fig. 14, where many tubules have a short dense spur projecting from one side. Following nuclear division, the median body elongates and may contribute to the formation of a second disc. If this is the case, the spur on each tubule profile probably marks the point of ribbon formation, and may contain giardin and directly nucleate giardin core assembly.

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REFERENCES


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