

Definition of individual components within the cytoskeleton of *Trypanosoma brucei* by a library of monoclonal antibodies

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Summary

The detergent-insoluble *T. brucei* cytoskeleton consists of several morphologically distinct regions and organelles, many of which are detectable only by electron microscopy. We have produced a set of monoclonal antibodies that define each structural component of this highly ordered cytoskeleton. The monoclonal antibodies were selected by cloning of hybridomas produced from mice injected with complex mixtures of proteins of either the cytoskeleton itself or salt extracts thereof. Four antibodies define particular tubulin isotypes and locate the microtubules of the axoneme and sub-pellicular

array; two antibodies recognize the flagellum attachment zone; one recognizes the paraflagellar rod and another the basal bodies. Finally, one antibody defines a detergent-insoluble component of the nucleus. The antigens detected by each monoclonal antibody have been analysed by immunofluorescence microscopy, immunogold electron microscopy and Western blotting.

Key words: *Trypanosoma brucei*, cytoskeleton, monoclonal antibodies.

Introduction

The plasma membrane forms the boundary of a cell and so defines its form: to achieve this the fluid mosaic must be moulded to the required shape and stabilized to permit selective positioning of membrane components and provide resistance to mechanical stress. This is achieved, at least in part, by an underlying cytoskeletal apparatus that interacts with the plasma membrane. In cells of higher eukaryotes, each of the three major cytoplasmic filament types (microfilaments, microtubules and intermediate filaments) can contribute to cell shape and stability: only rarely does one filament system perform this function exclusively. By contrast, in some eukaryotic microbes, particularly the free-living and parasitic flagellates, a single filament type (often microtubules) forms the 'membrane skeleton'.

African trypanosomes belonging to the *Trypanosoma brucei* complex are the causative agents of sleeping sickness in man and of nagana in livestock. This organism forms an ideal system for the investigation of the microtubule-based membrane-associated cytoskeleton. Procyclic forms are easily cultured *in vitro* and have a cytoskel-

etal structure that is highly ordered but simple enough to be experimentally accessible. In *T. brucei* there are discrete cytoskeletal structures in both the cell body and flagellum. In the cell body, a precisely ordered array of microtubules is closely associated with the plasma membrane (Angelopoulos, 1970). Electron microscopy has revealed the presence of cross-bridges connecting microtubules to the membrane and to each other (Vickerman, 1985). Curiously, there are no observable cytoskeletal microfilaments within the cytoplasm, even though trypanosomes apparently possess a functional actin gene (Amar *et al.* 1988). A specialized region of the membrane skeleton exists close to the site where the flagellum meets the cell body, namely, the flagellum attachment zone (FAZ). This appears as a zip-like structure in the electron microscope and has not yet been isolated or characterized biochemically. In the flagellum, in addition to a 9+2 microtubule axoneme, a paraflagellar rod is present. The paraflagellar rod of trypanosomes is antigenically related to that of *Crithidia* and *Euglena*, the major cross-reacting protein component appearing on SDS-containing gels as a doublet of M_r 68 000 and 76 000 (Russell *et al.* 1983; Gallo & Schrevel, 1985).

It appears that only a few cytoskeletal organelles, primarily based on microtubules, control the cell's shape and stability. Understanding the generation and maintenance of cytoskeletal order requires a detailed description of the use and positioning of particular gene products within the cytoskeleton. Although trypanosomes possess multiple tubulin genes (Seebeck *et al.* 1983), only one type of α - and one type of β -tubulin mRNA can be detected; nevertheless, a number of protein isoforms are produced by post-translational modification of the α -chain (acetylation of lysine 40 and detyrosination/tyrosination of the C terminus; (Steiger *et al.* 1984; Sasse & Gull, 1988). Their cellular distribution and modulation throughout the cell cycle has now been described (Sherwin *et al.* 1987; Sasse & Gull, 1988). The components of the cross-bridges between the cell body microtubules and the plasma membrane have not been biochemically defined (Bramblett *et al.* 1984; Vickerman & Preston, 1976); they do not seem to be transmembrane proteins and two lipid- and tubulin-binding proteins (M_r 40 000 and 60 000; Seebeck *et al.* 1988) seem to be the best candidates. The cross-bridges between microtubules have not yet been defined.

A major limitation on attempts to determine the biochemical cytology of the cytoskeleton is the availability of some of the components; the FAZ, for example, comprises only a small fraction of the cytoskeletal protein. Nevertheless, detergent-insoluble cytoskeletons that retain the shape and order observed *in vivo* may be isolated from trypanosome cells. Using these as complex immunogens, it should be possible to prepare monoclonal antibodies specific for individual components. These would identify components of structures otherwise inaccessible to standard biochemical analysis. Concomitantly, they would provide probes for the analysis of individual components during the cell cycle and their detection during attempts at purification.

In this paper we describe the preparation of a panel of nine monoclonal antibodies to cytoskeletal antigens. They provide novel probes for components of the FAZ, basal body, paraflagellar rod and nucleus as well as certain isotypes of tubulin.

Materials and methods

Cell lines

Procyclic *Trypanosoma brucei*, stock 427 were grown in tissue-culture flasks in semi-defined medium 79 (Brun & Schonberger, 1979). Vero cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal calf serum (FCS) and non-essential amino acids (NAA). PtK₂ cells were grown in Eagle's minimal essential medium supplemented with 10% (v/v) FCS and NAA.

Production of monoclonal antibodies

Mice were either immunized with detergent-insoluble cytoskeletons or salt extracts containing depolymerized pellicular microtubules. For the preparation of cytoskeletons cells were harvested by centrifugation, washed in phosphate-buffered saline (PBS) and then incubated on ice for 5 min in 0.5% Nonidet P40, 100 mM-Pipes, pH 6.9, 2 mM-EGTA, 1 mM-MgSO₄,

0.1 mM-EDTA (NP40/PEME). Cytoskeletons harvested from 1.5×10^8 cells, washed twice in PBS (0.14 M-NaCl, 2.5 mM-KCl, 8 mM-Na₂HPO₄, 1 mM-KH₂PO₄) and resuspended in 0.2 ml PBS, were used per injection per mouse.

For salt extracts cytoskeletons were resuspended in PEME, 1 M-KCl and left on ice for 40 min before centrifugation for 10 min at 11 600 *g* *av*. The supernatant was then dialysed against PBS. The extract from 5.5×10^8 cells was used per injection per mouse.

Balb/c mice were inoculated. For the initial inoculation the antigen was emulsified with an equal volume of Freund's complete adjuvant and applied intraperitoneally. Repeat injections were made in Freund's incomplete adjuvant. Final inoculations were done in PBS alone and administered intravenously. Splenectomy was performed 4 days after the final injection and the splenocytes were fused with approximately a third of their number of Sp2-O/Ag myeloma cells using polyethylene glycol (PEG) 1500, essentially as described by Galfre *et al.* (1977). Normal mouse splenocytes were used as feeder cells. A 100 μ l sample of medium was taken from each well between 14 and 21 days postfusion and screened for the presence of anti-cytoskeleton antibodies by immunofluorescence. Cloning of positive wells was performed by limiting dilution three times. Typing of the subclasses of each antibody was carried out by double immunodiffusion against specific antisera from ICN, Immunobiologicals, UK.

Immunofluorescence

Trypanosomes were washed in PBS and settled onto poly-L-lysine-coated slides. Slides were fixed in cold methanol for 30 min and rehydrated in PBS. Isolated flagella complexes were prepared according to Sasse & Gull (1988). Slides were incubated in culture supernatant for 1 h in a moist atmosphere and washed three times in PBS. The slides were then incubated with fluorescein-conjugated second antibody (rabbit anti-mouse immunoglobulins, Dakopatts) for 1 h and washed in PBS before mounting in Mowiol 488 (Harco, Harlow, UK) containing 1 mg ml⁻¹ *p*-phenylene diamine as an antifade agent.

Vero and PtK₂ cells (grown on coverslips) were washed in warmed PBS, fixed in cold methanol and incubated in antibodies, washed and mounted as above.

SDS-polyacrylamide gel electrophoresis

The method of Laemmli (1970) was used, with SDS obtained from Sigma (Clayton *et al.* 1980). The polypeptides were either stained with Coomassie Blue R250 or transferred to nitrocellulose paper.

Immunoblotting

With the exception of gels to be blotted and probed with CD10, proteins were transferred to nitrocellulose paper (45 μ m pore size, Schleicher & Schuell) probed with primary and secondary antibody (Dakopatts) as described by Birkett *et al.* (1985). Antibody supernatants were diluted with an equal volume of either 10 mM-Tris-HCl (pH 7.4), 140 mM-NaCl containing 0.1% (v/v) Tween 20 (TBS-Tween) in the case of Alphabet, C3B9 and PFR, or 10 mM-Tris-HCl (pH 7.4), 1 M-NaCl, 0.5% (v/v) Tween 20 (HST) in the case of TAT1, CD10 and CA12.

Proteins to be probed with CD10 were transferred to nitrocellulose according to Small (1988).

Immunogold

The method of Sherwin & Gull (1989) was used. Cells were harvested and washed in PBS. After settling onto Formvar-film, carbon-coated grids, they were then extracted using NP40/PEME. Cytoskeletons were then fixed in 3.7% parafor-

maldehyde, 0.01% glutaraldehyde, followed by washing in 20 mM-glycine in PBS. Grids were then blocked with 1% BSA (bovine serum albumin) in PBS and incubated in first antibody diluted in 1% BSA in PBS for 1 h at room temperature. After washing in 1% BSA in PBS (3 times, 5 min) the grids were incubated in second antibody (rabbit anti-mouse 10 nm gold conjugate, Biocell) diluted in 1% BSA in PBS for 1 h. Washes were then done in 1% BSA in PBS, followed by 0.1% BSA in PBS and then by a wash in PEME. Cells were then refixed in 2.5% glutaraldehyde and negatively stained using 2% ammonium molybdate, pH 7.0.

Synthetic peptides

Peptides corresponding to the *Physarum polycephalum* myxamoebal α -tubulin sequence residues 37–45 and the mammalian α -tubulin sequence residues 36–46 were made and kindly donated by Adrian Blindt and Paul Walden (this laboratory). A 2 mg sample of peptide (8 mg ml⁻¹ in water) was acetylated using acetic anhydride (Means & Feeney, 1971): 250 μ l of saturated sodium acetate was added to the peptide solution, then six additions, each of 4 μ l of acetic anhydride (10% in 1,4-dioxane), were made during a 1-h incubation on ice. The acetylated peptides were desalted on a G15 column using 50% acetic acid. The peak fractions containing each peptide were pooled. The acetic acid was removed by rotary evaporation, followed by methanol washes and the peptide residues were each resuspended in 1 ml of water to give a final concentration for each of approximately 1.5 mg ml⁻¹.

C3B9 culture supernatant was diluted 20-fold in TBS-Tween. The following combinations of antibody and peptides were then set up: (1) 2.5 ml C3B9 + 500 μ l acetylated peptide solution; (2) 2.5 ml C3B9 + approx. 95 μ l unacetylated peptide solution + 405 μ l water; (3) 2.5 ml C3B9 + 500 μ l water. The pH of each was adjusted to approximately 7.5 using 2 M-Tris-HCl and they were incubated overnight at 4°C with gentle agitation. The incubated antibody solutions were then used to immunostain identical Western blots of total *T. brucei* proteins separated by the normal one-dimensional PAGE method.

Preparation of erythrocytes and erythrocyte ghosts

Human or porcine blood was anticoagulated with EDTA. Washed erythrocytes and ghosts were prepared by the method of Bennett (1983).

Results

The *T. brucei* cytoskeleton

Detergent extraction of *T. brucei* cells reveals a cytoskeleton that retains the original cell shape and form. The major structural components of this cytoskeleton can be visualized by negative-stain electron microscopy. Fig. 1 shows such a cytoskeleton and acts as an ultrastructural reference for the light-microscope images described later. There are seven major features in the cytoskeleton of *T. brucei*. The major component of the cell body cytoskeleton is a helical arrangement of microtubules that are extensively cross-linked. The flagellum, subtended by a basal body located at the posterior of the cell, extends along the cell body, eventually overlapping the anterior end by a short distance. There are two major components within the flagellum: the microtubule axoneme and the paraflagellar rod, and both are clearly distinguished in these cytoskeletal preparations (Fig. 1). Also a probasal

body is located close to the flagellar basal body. Negative-stain electron microscopy of the *T. brucei* cytoskeleton permits the visualization of a complex periodic structure termed the flagellum attachment zone. This structure serves to link the flagellum to a specific region of the cortical microtubule array. Finally, the cytoskeleton preparations contain a specific identifiable remnant of the nucleus retained in its original cellular position (Fig. 1).

Selection of a panel of monoclonal antibodies

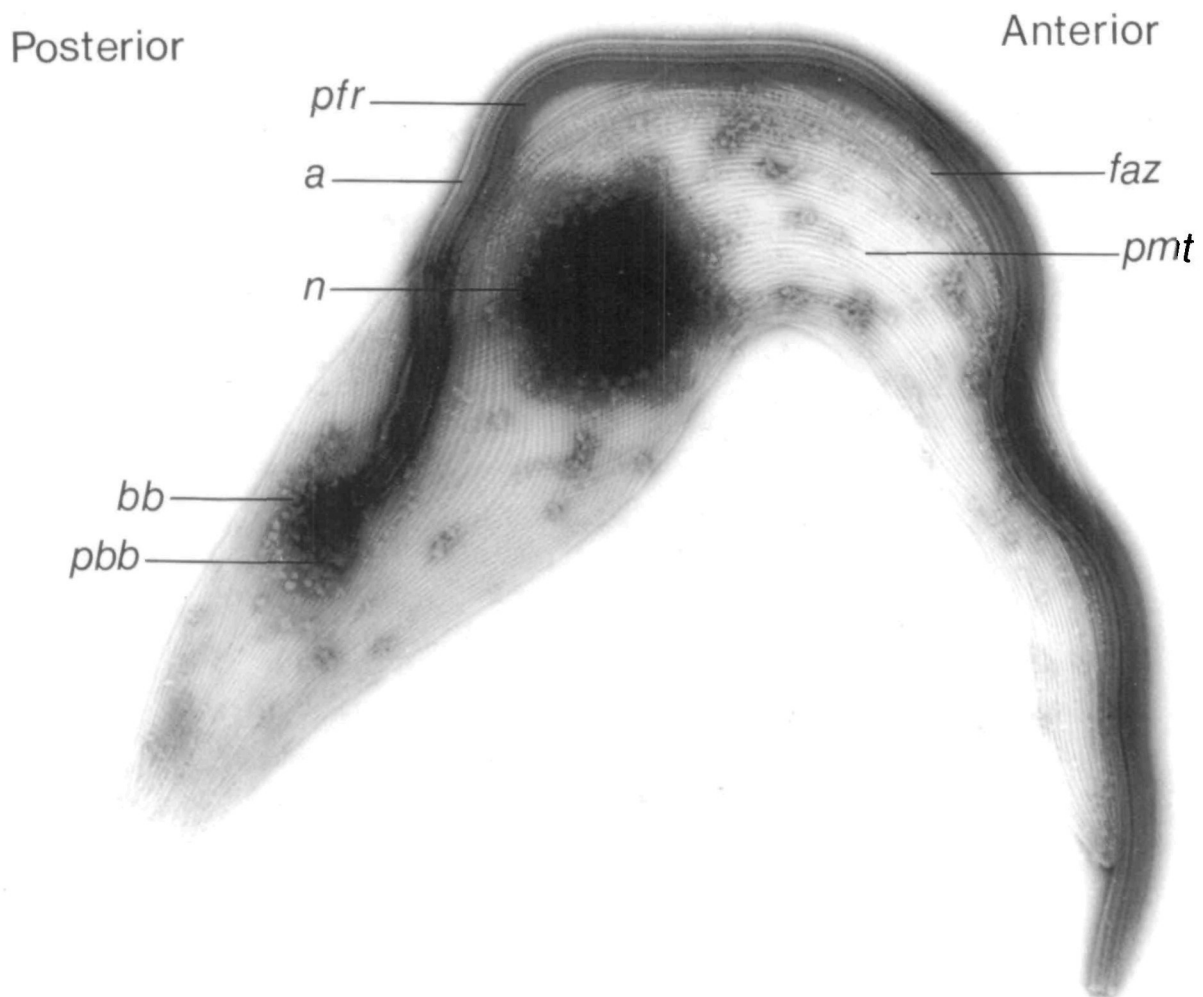
Purification of all the individual components of the complex *T. brucei* cytoskeleton is unrealistic. Consequently, in order to produce a panel of monoclonal antibodies recognizing specific individual components we used a complex immunogen and then used immunofluorescence subsequently to assay and categorize the antibodies. We used two separate immunogens: first, the complete, intact cytoskeleton; and second, the supernatant from a high-salt extraction of the cytoskeleton in order to produce a complex yet soluble immunogen. We selected and characterized hybridoma cell lines producing nine monoclonal antibodies. Table 1 surveys each of these antibodies and provides an overall summary of their nomenclature, class and specificity. This panel of antibodies provides probes for each of the major structural components of the cytoskeleton of *T. brucei* (Table 1 and Fig. 2).

Microtubules

This study produced four monoclonal antibodies (Alphabet, TAT1, C3B9 and CA12), each of which gave a general immunofluorescence recognition of the cell body together with the flagellar axoneme (Fig. 2). Immunogold electron microscopy shows that each recognizes both the pellicular and axonemal microtubules (Fig. 3). Western blotting revealed that all four monoclonal antibodies recognize the tubulin component of the microtubules, yet there are interesting and unusual differences in their

Table 1. Survey of monoclonal antibodies

| Antibody name | Immunogen | Antibody class | Specificity |
|---------------|-----------------------|----------------|---|
| Alphabet | Cytoskeletons | IgM | α - and β -tubulin |
| TAT1 | Cytoskeletons | IgG2b | α -tubulin |
| C3B9 | High-salt supernatant | IgG2b | Acetylated α -tubulin |
| CA12 | High-salt supernatant | IgG3 | β -tubulin (trypanosome-specific) |
| NUC | High-salt supernatant | IgG3 | Nucleus |
| BBA4 | High-salt supernatant | IgM | Basal body |
| ROD1 | Cytoskeletons | IgM | Paraflagellar rod (doublet, M_r 180+200K) |
| CD10 | High-salt supernatant | IgG3 | Flagellar attachment zone (M_r > 300K) |
| DOT1 | Cytoskeletons | IgM | Flagellar attachment zone and distal tip of flagellum |



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Fig. 1. Negatively stained electron micrograph of a detergent-insoluble cytoskeleton of *T. brucei*. Showing nucleus (*n*), basal body (*bb*), probasal body (*pbb*), the flagellar attachment zone (*faz*), the paraflagellar rod (*pfr*), pellicular microtubules (*pmt*) and the microtubular axoneme (*a*).

recognition epitopes. The monoclonal antibody Alphabet detects both α - and β -tubulin polypeptides of both *T. brucei* and mammalian brain microtubules (Fig. 4). TAT1 specifically recognizes α -tubulin and again cross-reacts with a wide range of species. This contrasts with CA12, which recognizes *T. brucei*, but not mammalian brain, β -tubulin. Thus, these three monoclonal antibodies illustrate the conservation of epitopes between the α - and β -tubulins and the evolutionary changes in the tubulin polypeptides leading to conserved and non-conserved regions. These anti-tubulin monoclonals should serve as useful probes for both the *T. brucei* cytoskeleton and the tubulin polypeptides.

The fourth monoclonal antibody that gave recognition of microtubules by immunogold electron microscopy, C3B9, recognized the α -tubulin of *T. brucei* when used in Western blotting experiments but detected mammalian brain α -tubulin to a limited extent only (Fig. 4). This result might be explained by a reduced general avidity for mammalian α -tubulin or, rather, that C3B9 recognizes an isotype of α -tubulin that is well-represented in the *T. brucei* microtubules but only poorly so in the mammalian brain tubulin. This latter explanation was supported by the results of immunofluorescence studies with the anti-

body. When Vero cells in culture were stained with the anti- α -tubulin monoclonal TAT1, the expected complex pattern of cytoplasmic microtubules was detected. However, C3B9 gave a very restricted immunofluorescence pattern of microtubules in Vero cells (Fig. 5). This antibody detects a sub-population of microtubules that have wavy profiles and a general perinuclear distribution. C3B9 also detects specific microtubule structures such as centrioles and mid-bodies. This immunofluorescence pattern is extremely similar to that of acetylated α -tubulin distribution in cells detected by other monoclonal antibodies (e.g. 6-11-B1; Piperno & Fuller, 1985). We have shown previously that the *T. brucei* cytoskeleton is rich in acetylated α -tubulin (Sasse & Gull, 1988). In order to assess whether C3B9 recognizes acetylated α -tubulin we subjected a preparation of mammalian brain tubulin to chemical acetylation. This resulted in a large increase in the detection of α -tubulin by C3B9 in comparison to an unacetylated control. The only known post-translational acetylation site on α -tubulin is a lysine residue at position 40 in the polypeptide. In order to assess whether this particular lysine is acetylated in *T. brucei* tubulin and to provide the recognition epitope for C3B9, we synthesized two peptides that span this region.

These corresponded to a consensus mammalian α -tubulin sequence (residues 36–46) and that of *P. polycephalum* α -tubulin (residues 37–45). Both peptides included the single lysine at residue 40.

We then used these peptides in both the unacetylated and acetylated forms in competition experiments with the C3B9. Fig. 6 shows the result of this experiment and

illustrates the fact that the peptide can compete out the binding of C3B9 to *T. brucei* α -tubulin, but only when the lysine in the peptide is in the acetylated form. Thus, C3B9 recognizes an epitope that includes an acetylated lysine at residue 40 in the α -tubulin polypeptide and is thus able to detect acetylated α -tubulin in an evolutionarily diverse range of organisms. Moreover, it provides

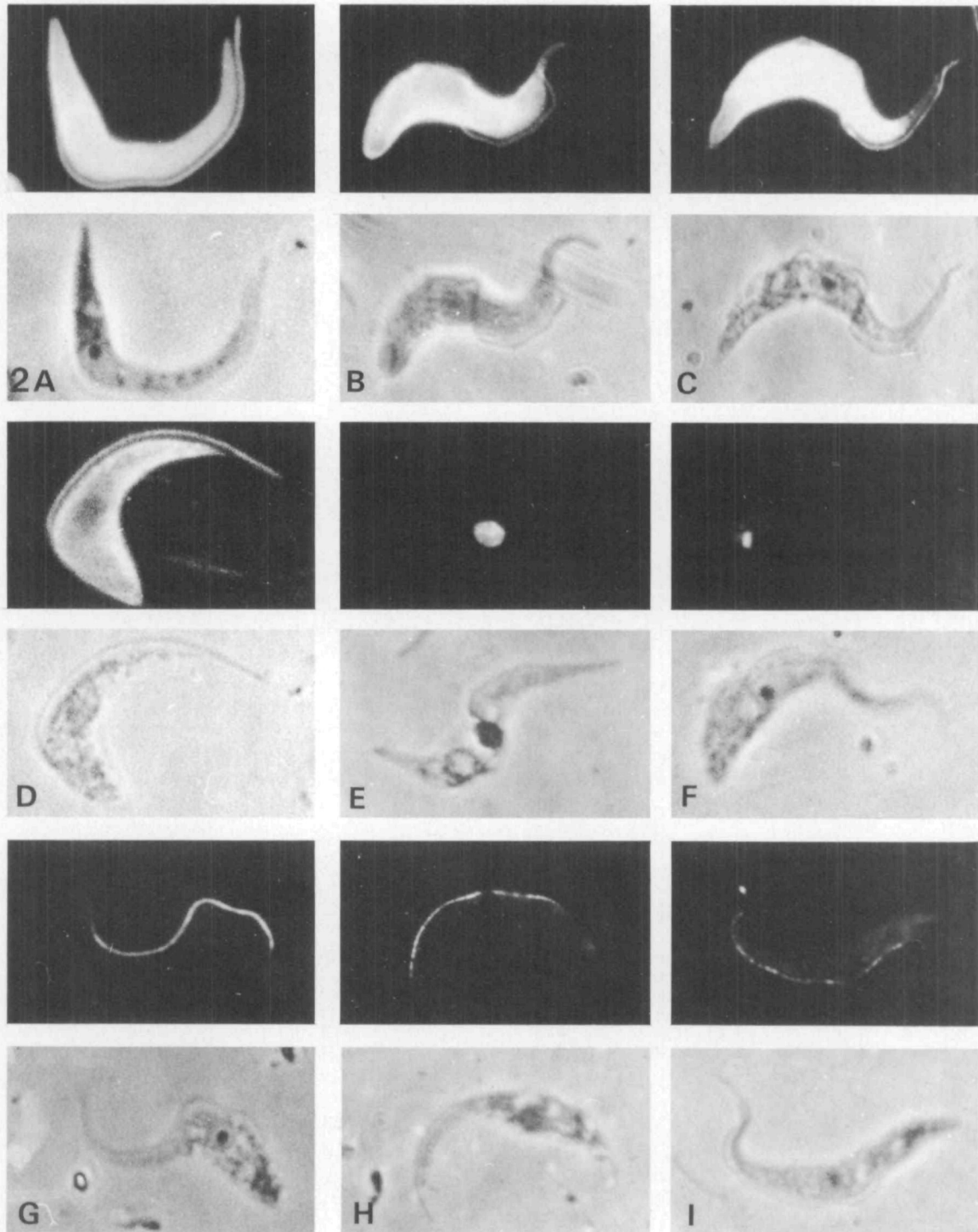


Fig. 2. Fluorescence (above) and phase-contrast (below) images of cells probed with different monoclonal antibodies. A. Alphabet; B, TAT1; C, C3B9; D, CA12; E, NUC; F, BBA4; G, ROD1; H, CD10; I, DOT1.

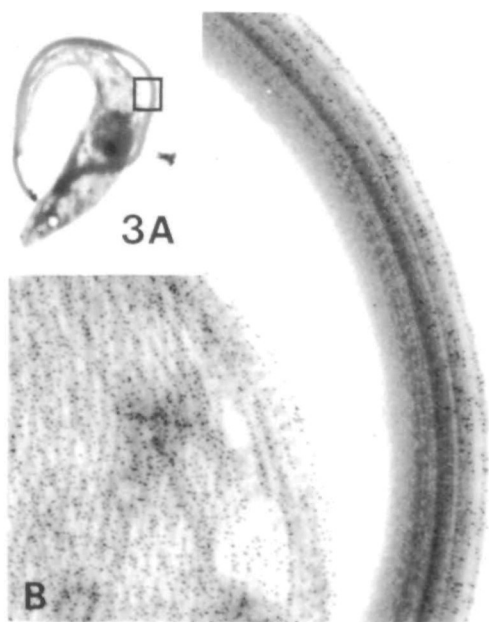


Fig. 3. Electron micrograph of immunogold staining of a cytoskeleton probed with TAT1. B shows enlarged region of cell, shown in a box in A.

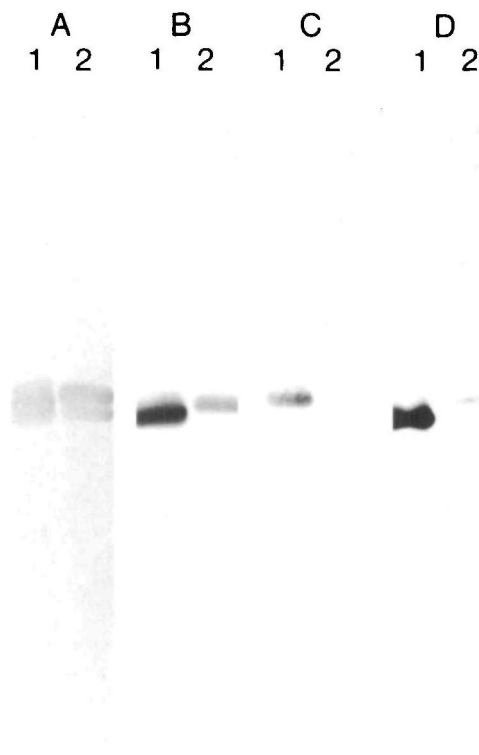


Fig. 4. Western blots using anti-tubulin monoclonal antibodies. Lanes 1, *T. brucei* whole-cell lysate; lanes 2, mammalian brain tubulin probed with Alphabet (A), TAT1 (B), CA12 (C), C3B9 (D). Note: gels run with buffers made using sigma SDS, therefore trypanosome α -tubulin migrates further than β , but in mammalian brain tubulin the reverse is true (Clayton *et al.* 1980).

direct confirmation of the presence of acetylated α -tubulin in subpellicular and axonemal microtubules in the *T. brucei* cytoskeleton.

The nucleus

The monoclonal antibody NUC is a representative antibody that detects the nucleus of *T. brucei* cells. This specific detection of the nucleus is shown in Fig. 2 and the immunofluorescence pattern does not appear to change with the position of the nucleus within the cell cycle. The antibody does not appear to be directed against DNA, since the large amount of kinetoplast DNA in the mitochondrion is not seen by immunofluorescence (Fig. 2). However, we have been unsuccessful in our attempts to identify a polypeptide recognized by NUC using various Western blotting protocols.

The basal bodies

BBA4 represents a monoclonal antibody that, when used in immunofluorescence assays, detects two very small structures towards the posterior portion of the *T. brucei* cell (Fig. 2). Phase/immunofluorescence images of cytoskeletons and isolated flagella stained with this monoclonal clearly show that it detects the flagellar basal body and pro-basal body (Fig. 7). We have been unable to detect recognition of a polypeptide using BBA4 by Western blotting experiments. It may be that recognition of the epitope is dependent on conformation or it is possible that the antigen is present in very small amounts. Nevertheless, BBA4 represents a specific probe for the basal bodies of the *T. brucei* cytoskeleton. The pattern of fluorescence detection emphasizes that the antigen detected by BBA4 becomes associated with the basal body very early during its biogenesis as a pro-basal body. Moreover, the antigen has an extremely restricted location within the cell and does not appear to be associated with either the flagellar axoneme or other cellular organelles. From staining patterns observed using the DNA intercalating dye, DAPI, it is apparent that BBA4 staining is distinct from the kinetoplast. Moreover, immunogold studies suggest that BBA4 does not react with a specific mitochondrion [kinetoplast]/basal body connection. BBA4 does not stain the basal bodies in PtK₂ cells, Vero cells or *Physarum* amoebae in fluorescence studies.

The paraflagellar rod

One monoclonal antibody, ROD1, when used in immunofluorescence studies provides a specific detection of the *T. brucei* flagellum (Fig. 2). Immunogold electron microscopy shows that ROD1 binds to the paraflagellar rod (Fig. 8C). Further, the antigen detected by ROD1 is located on the area of the paraflagellar rod closest to the zone of attachment to the cell body (Fig. 8). The major constituents of the paraflagellar rod are polypeptides with molecular weights of 76K and 68K ($K = 10^3 M_r$) (Russell *et al.* 1983). One monoclonal antibody, 5E9, has already been shown to recognize both of these polypeptides (Gallo & Schrevel, 1985). Western blot analysis of ROD1 revealed that this monoclonal did not detect the major PFR1 and PFR2 proteins but, rather, a doublet of two

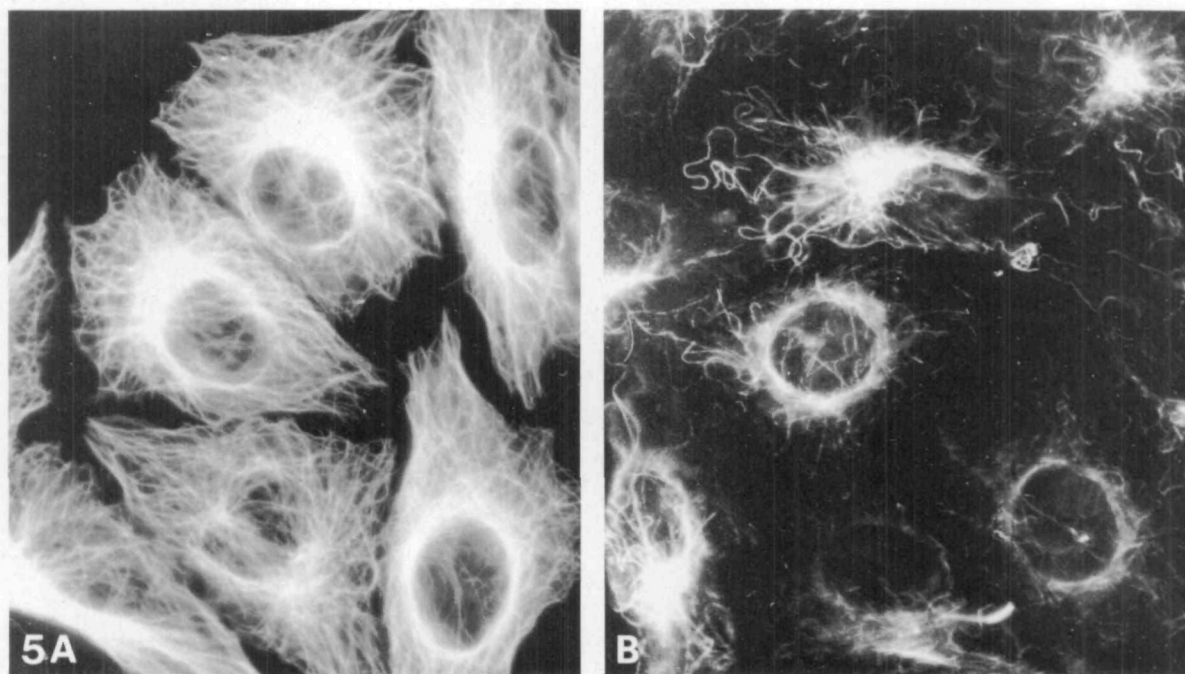


Fig. 5. Immunofluorescence staining pattern of Vero cells probed with TAT1 (A) and C3B9 (B).

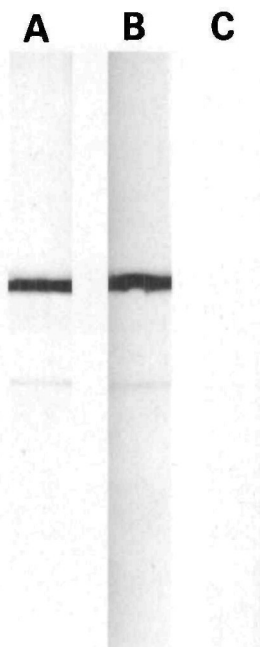


Fig. 6. Competitive binding of C3B9 and peptides on identical Western blots. A. C3B9 supernatant; B, C3B9 supernatant previously incubated with unacetylated peptide; C, C3B9 supernatant previously incubated with acetylated peptide.

polypeptides with molecular weights of around 180K and 200K (Fig. 8).

When used to probe Western blots of human and porcine erythrocyte polypeptides, ROD1 detects bands of slightly higher molecular weight that comigrate with both human and porcine β -spectrin. ROD1 also gives a

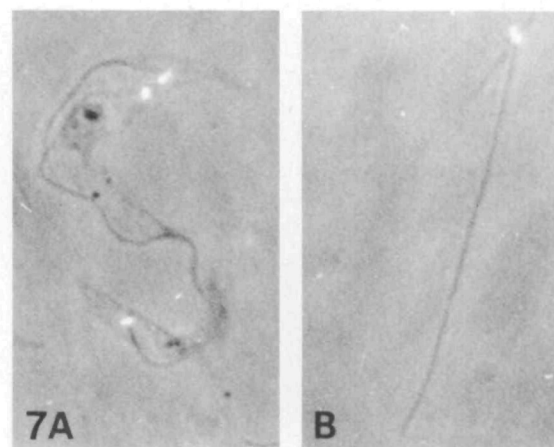


Fig. 7. Immunofluorescence/phase-contrast pattern of BBA4 on a post mitotic cell (A), and an isolated flagellum (B).

general whole-cell staining pattern when used in immunofluorescence assays with human erythrocytes (not shown).

The flagellum attachment zone

Two monoclonal antibodies, CD10 and DOT, gave immunofluorescence staining of *T. brucei* cells that indicated that they both detected components of the flagellum attachment zone (Fig. 2). CD10 detects an intermittently punctate line along the flagellum attachment zone. Immunogold electron microscopy revealed that the antigen detected by CD10 is located along a complex filament system that comprises the FAZ (Fig. 9). Further, immunofluorescence studies show that this filament system is

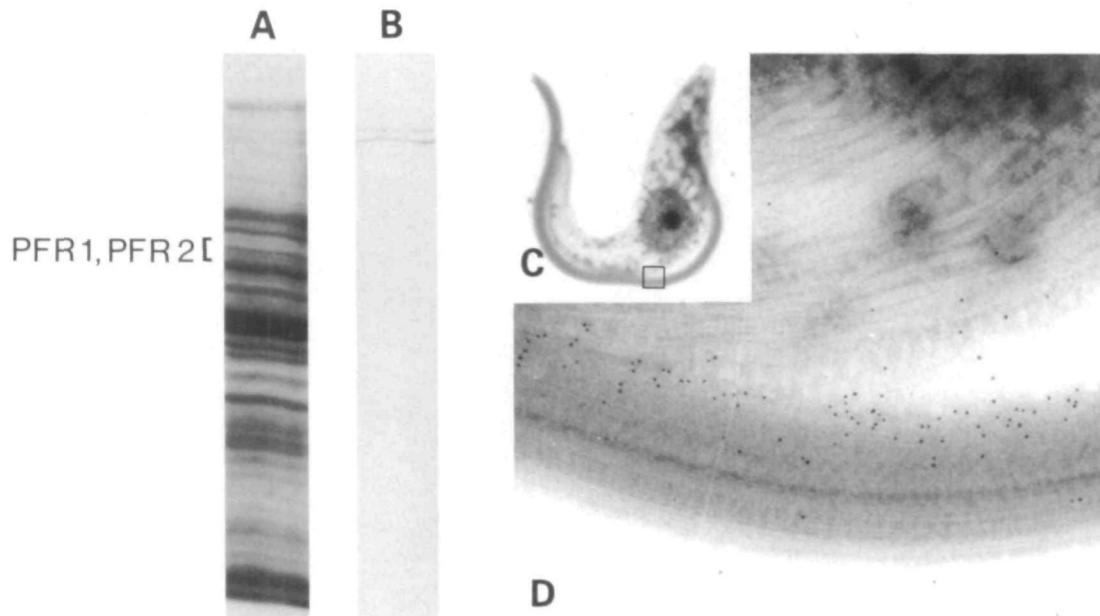


Fig. 8. A and B show corresponding Coomassie Blue-stained gel and Western blot probed with ROD1. A, Coomassie Blue-stained gel of *T. brucei* whole-cell lysate; and B, corresponding Western blot probed with ROD1. D shows enlarged area of the cytoskeleton, shown by a box in C, which has been immunogold labelled with ROD1.

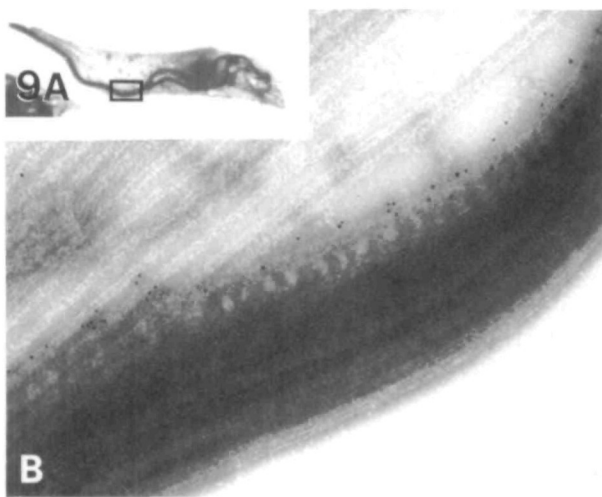


Fig. 9. Electron micrograph of immunogold staining pattern using CD10. B shows enlarged area of the cytoskeleton, shown in a box in A.

elaborated during the process of flagellar growth. Cells late in the cell cycle, which possess two flagella, have two flagellum attachment zones revealed by the CD10 monoclonal. Western blotting of *T. brucei* proteins using CD10 (Fig. 10) revealed that this monoclonal detects a polypeptide of very high molecular weight (>300K). The detection of the CD10 antigen relies upon using a particular blotting protocol that was developed for use with integral membrane proteins.

The DOT1 monoclonal antibody gives an immunofluorescence image on *T. brucei* cells that is superficially similar to that of CD10. There are, however, subtle differences in that DOT1 gives a more punctate pattern

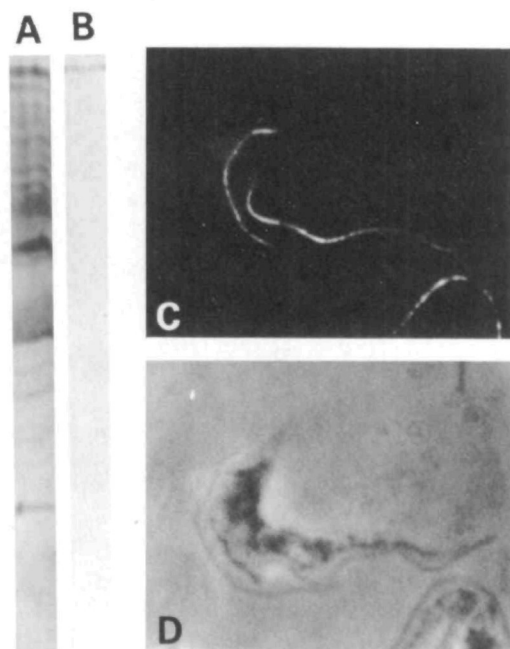


Fig. 10. A shows the total protein of whole-cell lysate, Amino Black-stained. B shows an identical blot immunostained with CD10. C shows the fluorescence staining pattern of a post-mitotic cell stained with CD10 (shown in phase-contrast in D).

and also detects material at the distal tip of the flagellum. So far we have been unable to detect an antigen for the DOT1 monoclonal antibody by exhaustive Western blot analyses. Immunogold electron microscopy of *T. brucei* cytoskeletons using DOT1 reveals that the antigen detected is located in regions associated with the unique

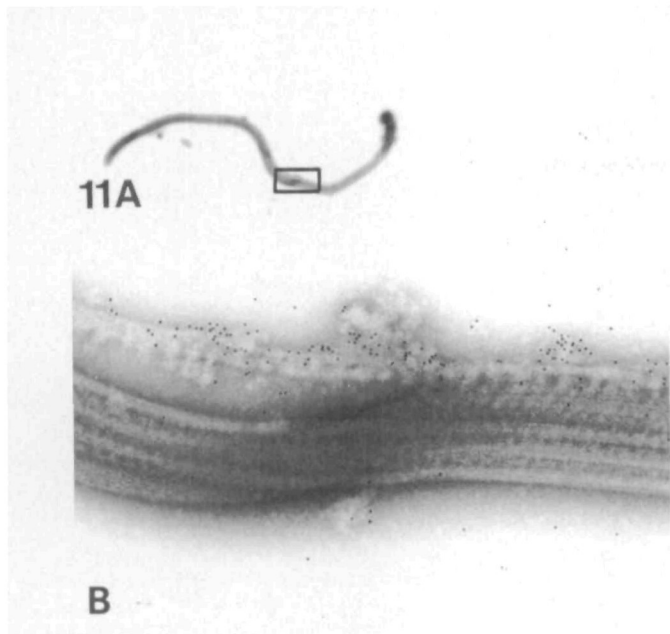


Fig. 11. Immunogold staining pattern revealed by DOT1 on isolated flagellum. B shows enlarged boxed area in A.

filament systems of the flagellum attachment zone (Fig. 11).

Discussion

The *T. brucei* cytoskeleton is very well defined ultrastructurally but the correlative biochemistry is underdeveloped. We have extensive information pertaining to the abundant molecules in the cytoskeleton, i.e. α - and β -tubulin and the PFR proteins (Sasse & Gull, 1988; Steiger *et al.* 1984; Schneider *et al.* 1987; Gallo & Schrevel, 1984). However, the biochemical identification of other cytoskeletal components is more problematical, since they resist classical approaches because of their insolubility or low abundance. Thus, we have adopted the approach of producing probes for structures and constituent molecules in the form of monoclonal antibodies produced against antigenic determinants present in a complex mixture of proteins, i.e. the cytoskeleton itself.

This has a number of advantages. Preparation of the immunogen is very easy, merely involving the production of detergent-insoluble cytoskeletons or salt extracts thereof. The use of immunofluorescence as an assay has enabled us to select antibodies that elicit a specific pattern in the cell, giving us the polypeptide localization from simple primary screens. This method of screening also permits detection of very small signals, which may present only minor components of the whole structure and may not be detectable by other screening methods such as ELISA or blotting techniques. This is exemplified by the BBA4 antibody.

The variety of tubulin antibodies obtained is not surprising, as tubulin is the most abundant single protein in the cytoskeleton of *T. brucei*. The fact that the four tubulin antibodies cross-react to differing extents between both species and tubulin isotypes demonstrates several properties of tubulin. TAT1 emphasizes the

evolutionary conservation of tubulin between species in that it cross-reacts with mammalian, *Physarum* and trypanosomal α -tubulin. The overall sequence homology between mammalian and trypanosomal α -tubulin is 84–85% (Kimmel *et al.* 1985). Alphabet shows that α - and β -tubulin share homologous antigenic regions, presumably corresponding to a portion of the approximately 40% of their amino acid sequence that is homologous. C3B9 is an example of an isoform-specific antibody, which enables the distribution of such post-translationally modified tubulin as well as its role in the cell cycle and tubulin dynamics to be studied. This antibody has also enabled us to demonstrate the evolutionary conservation of lysine 40 as an acetylation site. CA12, however, appears to be specific. It does not cross-react with tubulin from any other species including *Physarum* (plasmodial or amoebal), *Chlamydomonas* or PtK₂ cells on Western blots. It was even found not to cross-react with tubulin from *Trypanosoma lewisi*, implying divergence of β -tubulin even within *Trypanosoma*. Each of these anti-tubulin antibodies will be useful probes in future experiments.

The flagellar attachment zone observed by thin-section electron microscopy appears as a complex, filamentous system and a series of punctate connections between the flagellar and pellicular membranes (Sherwin & Gull, 1989). There are invariably four microtubules associated with a portion of the smooth endoplasmic reticulum, which lie immediately to the left-hand side of the flagellar attachment zone when viewed from the posterior of the cell (Gallo & Precigout, 1988). Our investigation, using monoclonal antibodies, has revealed two novel proteins exhibiting differing properties, which are located in this complex structure. These antibodies used in immunofluorescence and immunogold studies show that the flagellar attachment zone acts to connect the flagellum to the cell surface as soon as a new flagellum has emerged from the flagellar pocket. Thus, development of the flagellum attachment zone appears to be concomitant with both axoneme and paraflagellar rod elongation.

The ROD1 monoclonal has revealed a doublet of polypeptides (M_r 180–200K), in addition to the previously described doublet, PFR1 and PFR2 (M_r 68–76K; Russell *et al.* 1983), to be present in the paraflagellar rod. These proteins are seen to be only minor components of the paraflagellar rod in comparison to PFR1 and PFR2. ROD1 has been shown to cross-react with a band that comigrates with β -spectrin in human and porcine erythrocyte ghosts. Schneider *et al.* (1988) reported that a polyclonal antibody raised against human erythrocyte spectrin cross-reacted with a doublet of 180–200K in *T. brucei*. It has been shown that spectrin is a protein not restricted to vertebrates but may indeed be ubiquitous, having been found in many tissue types and evolutionarily diverse organisms (Baines, 1984; Virtanen *et al.* 1984; Pollard, 1984). A recent report by Schneider *et al.* (1988) has shown that antibodies raised against mammalian spectrin cross-react with a trypanosome protein in the paraflagellar rod. Conversely, we have now raised an antibody to trypanosome proteins that cross-reacts with mammalian spectrin.

The trypanosome cell undergoes a variety of changes in cell shape and form during its life cycle. These changes are likely to be due to the direct modulation of its intracellular cytoskeleton. Moreover, the complex flagellum and flagellar attachment zone is undoubtedly involved in the establishment of connections involved in attachment of the *T. brucei* cell to host cell surfaces in the *Glossina* stage of its life cycle. Our library of monoclonal antibodies, which defines each component of the cytoskeleton, should prove useful in future studies of these intra- and inter-cellular interactions.

This work was supported by grants from the Science Engineering Research Council, Medical Research Council (and The Royal Society). This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. We thank Adrian Blindt and Paul Walden for very kindly providing the peptides.

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(Received 27 January 1989 – Accepted 6 April 1989)