Disruption of microtubules in living cells and cell models by high affinity antibodies to beta-tubulin

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Polyclonal antibodies with high affinity for β -tubulin were found to disrupt cytoplasmic microtubules efficiently after microinjection into tissue culture cells. The degree of microtubular fragmentation was directly proportional to the amount of the injected antibody. At molar ratios of 1 antibody per 100 tubulin dimers, most microtubules were disrupted within 90 min after injection. In contrast, the time course of disintegration was relatively independent of the antibody concentration. Within the range of 1 antibody per 10^2 – 10^4 tubulin dimers, the maximal values for microtubular disintegration were reached ~ 1 – 1.5 h after injection. Mitotic microtubules were found to be resistant to all antibody concentrations used. In living cells, microtubules recovered within a few hours after antibody-induced decay. The time course of recovery, like the extent of disintegration, was a function of the antibody concentration. The antibody acted also on microtubules in detergent-extracted cell models and on microtubules polymerised *in vitro*. When added to microtubular protein, the bivalent antibody as well as its Fab fragments prevented polymerisation. The data suggest that these antibodies disrupt microtubules because their affinity to tubulin is at least 100 times higher than the affinities found for tubulin:tubulin interaction. Fragmented microtubules are probably unstable and decompose into smaller units.

Key words: β -tubulin-specific antibody/microinjection/antibody-induced microtubular disruption

Introduction

Microinjection of antibodies into tissue culture cells has recently developed into an attractive method to study structure and function of complex cytoskeletal elements such as microtubules and microfilament bundles (for reviews, see Taylor and Wang, 1980; Kreis and Birchmeier, 1982; Jockusch and Füchtbauer, 1985; Jockusch *et al.*, 1985). Specific polyclonal as well as monoclonal antibodies prepared against structural proteins from muscle or non-muscle tissue cross-react with the corresponding antigen in tissue culture cells due to the highly conserved nature of many cytoskeletal components. Introduced into the living cell by microinjection and bound in antigen-antibody complexes, their action can be followed either directly (if the antibodies had been labeled with fluorescent marker molecules) or indirectly by labeled second antibodies.

The microinjection of anti-tubulins into tissue culture cells has recently been reported (cf. Wehland and Willingham, 1983; Blose

et al., 1984). Regarding the consequences of introducing such antibodies into the cell, one can imagine several possibilities.

(i) The antibody may interfere with certain microtubular functions, but not with microtubular integrity and cellular distribution. This has been found for a rat monoclonal antibody against tyrosylated α -tubulin (Kilmartin *et al.*, 1982; Wehland *et al.*, 1983). When complexed with colloidal gold particles and injected into PtK₂ cells in low concentrations (16 μ g/ml), the antibody blocked the saltatory movement of such particles along individual microtubules (De Brabander *et al.*, 1985).

(ii) Bivalent or multivalent antibodies may block several microtubule-dependent motility processes, by aggregating microtubules. Such an effect was observed, when rather high concentrations (6 mg/ml and above) of the same antibody were injected. Saltatory movement of endogenous organelles and chromosomal separation were inhibited in the injected PtK₂ cells (Wehland and Willingham, 1983).

(iii) The antibody may interfere with cellular architecture by disrupting links between microtubules and other cytoplasmic structures. Such cases were described for very high concentrations (10–20 mg/ml) of several antibodies: the intermediate filament network collapsed into perinuclear caps, and the Golgi apparatus disintegrated into dispersed vesicles (Wehland and Willingham, 1983; Blose *et al.*, 1984).

(iv) The antibody may interfere with microtubular integrity, by binding to epitopes on the tubulin molecule which are essential for microtubule polymerisation or maintenance of structure. In this case, the principal effect should be similar within a wide range of antibody concentrations.

Here, we describe experiments which suggest that the anti-tubulin used is of the last kind. It is a polyclonal antibody of high affinity prepared against porcine brain tubulin in rabbits, purified by affinity chromatography and selected for β -tubulin specificity by cross-absorption (Herrmann, 1981; Mandelkow *et al.*, 1985). This antibody disrupts reversibly cytoplasmic microtubules after being microinjected in various concentrations. It severs microtubules in cell models as well as microtubules polymerised *in vitro*. Part of this study has been presented in preliminary form (Herrmann *et al.*, 1982; Mandelkow *et al.*, 1983).

Results

As seen by indirect immunofluorescence, the antibodies with high affinity for β -tubulin decorated cytoplasmic microtubules homogeneously. In mitotic PtK₂ cells, spindle fibers, spindle poles and small, punctate patches at the plasma membrane were stained (Figure 1A,B). To visualise the effect of such antibodies on living cells, we used two different procedures. In one, we employed the same antibody for injection and staining of cells. For this purpose, we had to quench the injected antibody before staining by coating it with unlabeled second antibody (see Material and methods for details). In the second protocol, we used a different antibody for staining injected cells. While the injected one had a high affinity for β -tubulin, the antibody used for staining would

Fig. 1. Staining of interphase, mitotic and fragmented microtubules in PtK₂ cells and in fibroblasts with anti- α - or anti- β -tubulin. **(A,B)** Cytoplasmic microtubules in interphase **(A)** and mitotic **(B)** PtK₂ cells, as visualised with the specific anti- β -tubulin and affinity-purified, rhodamine-coupled sheep-anti-rabbit IgG (Rh-SaR). Note the patches at the periphery of the mitotic cell (arrows), indicating the presence of β -tubulin at the plasma membrane. **(C,D)** Fragmentation of cytoplasmic microtubules after the injection of anti- β -tubulin. PtK₂ cells were injected with 1 mg/ml antibody (arrowheads) and fixed 30 min later. **(C)** The injected antibody was quenched with unlabeled goat anti-rabbit IgG (GaR), the cells were then stained with anti- β -tubulin followed by Rh-SaR. **(D)** Cells stained with the monoclonal anti- α -tubulin YL 1/2 (Kilmartin *et al.*, 1982) followed by rhodamine-coupled rabbit anti-rat IgG (Rh-RaRt). In both cases, fragmentation of microtubules as a consequence of anti- β -tubulin injection is clearly visible. **(E,F)** Details of non-injected WI 38 fibroblasts stained with anti- β -tubulin. **(E)** Normal indirect immunofluorescence, as in **(A)**. **(F)** Indirect immunofluorescence after treatment of the fixed cell with unlabeled goat anti-rabbit IgG, according to the quenching protocol. Bars: **(A,C,D)**, 20 μ m, **(B)**, 10 μ m, **(E,F)**, 20 μ m.

decorate exclusively the tyrosylated form of α -tubulin (see Wehland and Willingham, 1983). With both procedures, it was clearly seen that cytoplasmic microtubules were fragmented by injection of the antibody against β -tubulin (Figure 1C,D). The treatment of fixed cells with unlabeled goat-anti-rabbit IgG followed by anti- β -tubulin, as used in the 'quenching procedure', did not interfere with a homogeneous decoration of microtubules

by the anti- β -tubulin: non-injected control cells, processed according to this protocol, displayed evenly stained microtubules, quite similar to those seen in normal indirect immunofluorescence (Figure 1E,F). The injection of control proteins like bovine serum albumin (BSA, Figure 2A,F) or affinity-purified anti-actin (not shown) had also no effect on the appearance of cytoplasmic microtubules.

Fig. 2. Disintegration and recovery of cytoplasmic microtubules in PtK₂ and WI 38 cells injected with anti- β -tubulin. (A–E) Cells quenched with unlabeled GaR and stained with anti- β -tubulin followed by Rh-SaR after injection (cf. Figure 1C,F). (F–I) Cells stained with the anti- α -tubulin YL 1/2 followed by Rh-RaRt (cf. Figure 1D). (A,F) Control PtK₂ cells injected with 1 mg/ml BSA in PBS, fixed 1.5 h after injection. (B,G) Cells injected with 1 mg/ml anti- β -tubulin in PBS (arrowheads), fixed 30 min after injection. (C,H) Cells injected with 1 mg/ml anti- β -tubulin in PBS (arrowheads), 50 (C) and 60 (H) min after injection. At this time point, disintegration of microtubules is almost complete. The injected cells are filled with amorphous aggregates. (D) WI 38 fibroblast injected with 1 mg/ml anti- β -tubulin (arrowhead), stained 4 h after injection. (E) Details taken at higher magnification from the cell in (B), to show microtubular fragments. (I) Cell injected with 1 mg/ml anti- β -tubulin in PBS (arrowhead), 5 h after injection. Microtubules have regrown. Bars: (A–C,F–I), 20 μ m, (D,E), 10 μ m.

Fig. 3. Time course of microtubular disintegration and recovery after microinjection of 1 mg/ml anti- β -tubulin into PtK₂ cells. Integrity of microtubules was measured using an average 'integrity index' ranging from 1 to 10, from homogeneously stained 'typical' microtubules only (integrity index 10) to unstructured, amorphous aggregates without discernible microtubular fragments or fibers (integrity index 0). Within this scale, a cell like the one shown in Figure 2G would score as '4', cells depicted in Figure 2C would score as '1'. Each point represents the average of 100 cells. Even after 21 h, the values have not fully returned to the original level which probably reflects the fact that some cells do not survive the microinjection process (cf. Füchtbauer *et al.*, 1983).

In a series of microinjection experiments, we found that the effect of the anti- β -tubulin on cytoplasmic microtubules was concentration- and time-dependent. Figure 2 shows examples of such experiments, where the cross-absorbed, β -specific antibody was used for injection and staining (Figure 2B,C). Alternatively, the anti- β -tubulin was injected, and the anti- α -tubulin was used for staining (Figure 2, G–I). Already 5 min after injection of antibody at 1 mg/ml, cytoplasmic microtubules could be seen which showed discontinuous staining probably due to breaks in the tubulin polymers (not shown). At 30 min p.i., most microtubules were fragmented (Figure 2B,G). The fragments varied in length and were spaced apart by unstained regions also variable in length (Figure 2E). The number of fragments increased with time in parallel with the appearance of amorphous aggregates, so that at 50–60 min after injection almost no intact microtubules could be seen but a fibrous precipitate was present throughout the cell (Figure 2C,H). Similar data were obtained with mouse mammary epithelial cells as well as with human WI 38 (Figure 2D) and primary fibroblasts from mouse and rat, indicating that this result was not due to a lability of cytoplasmic microtubules specific for PtK₂ epithelial cells.

When the injected cells were incubated for longer time periods before fixation, it became obvious that the cells were able to recover from this effect of intracellular anti-tubulin. Figure 2I shows a PtK₂ cell injected with 1 mg/ml anti- β -tubulin and processed for immunofluorescence 5 h later. Many microtubules are seen in the perinuclear area and several long microtubules extend from this region to the periphery of the cell. Examination of cells at various time points indicated that recovery started with the appearance of many short microtubules within the perinuclear region, while longer microtubules appeared later. This observation may suggest that recovery includes the outgrowth of new microtubules from the microtubule organising center.

While the disintegration of microtubules started at multiple sites within a few minutes after introducing the antibody and was complete after 1–2 h, the recovery process took much longer. Figure 3 demonstrates the time course of microtubular disintegration and recovery in PtK₂ cells injected with 1 mg/ml antibody and processed for immunofluorescence over a period of 21 h.

Fig. 4. Microtubular recovery after anti- β -tubulin-induced disintegration as a function of antibody concentration. These values have been deduced from single graphs analogous to Figure 3. The points correspond to the means of those positions where in the original graphs the curves flatten out and approach the highest level of restored microtubules. The vertical bars indicate the variations between different experiments. For each value, 300–5000 cells were scored.

The time period required for recovery of cytoplasmic microtubules was inversely correlated with the concentration of the injected antibody, as seen in Figure 4. This figure shows also that a transient effect within 0.5–1.5 h was seen with concentrations as low as 10 μ g/ml. At such low concentrations, disintegration of microtubules usually proceeded as rapidly as with higher concentrations (cf. Füchtbauer, 1984), but only to the stage of distinct fragmentation, while formation of fibrous or amorphous aggregates was limited.

Mitotic microtubules were more resistant to antibody attack than cytoplasmic ones. Within the asynchronous cultures we used, prophase and early metaphase cells were identified in phase contrast optics. They were subsequently injected with anti- β -tubulin (1 mg/ml) and fixed at different times after injection (0.5, 1, 2, 3, 4 and 5 h p.i., at least 30 cells for each time point). Intact spindle fibers were seen early after injection. At 2 h and later p.i., injected cells were seen in anaphase, telophase and various stages of cytokinesis. Our experiments cannot exclude a short delay in either spindle assembly or chromosome separation as a consequence of anti-tubulin injection. However, we can conclude that injected cells were able to proceed through mitosis and divide normally.

The antibody was also capable of breaking down microtubules in detergent-treated cell models. This is demonstrated in Figure 5. PtK₂ cells and fibroblasts (not shown) were incubated in a microtubule stabilising extraction buffer containing polyethylene glycol, GTP and Triton X-100 (Osborn *et al.*, 1978). In this buffer, cytoplasmic microtubules appeared unaltered for up to 3 h, as judged by subsequent fixation and processing for indirect immunofluorescence (Figure 5A). However, when anti- β -tubulin was added, microtubules were disrupted within minutes. With 0.1 mg/ml antibody, severe damage was already obvious after 5 min incubation time (Figure 5B) and progressed rapidly until no intact microtubules could be discriminated ~30 min later (Figure 5C). The time course and extent of microtubular breakdown in cell models as a function of the antibody concentration are shown in Table I. As with intact cells, spindle microtubules of detergent-extracted mitotic cells were highly resistant to antibody-induced breakdown (Figure 5C, inset).

When microtubule protein from porcine brain was incubated with the complete antibody or Fab fragments at low temperature (4°C) prior to assembly, a concentration-dependent inhibition of

Fig. 5. Anti- β -tubulin-induced microtubular breakdown in detergent-extracted cell models. Cells were extracted with 0.2% Triton X-100 in a microtubule stabilising buffer (MSB). (A) Control cells left for 3 h in MSB. (B) cells incubated with 0.1 mg/ml antibody in MSB for 5 and (C) for 30 min prior to fixation and processing for immunofluorescence using the same antibody. Microtubules in (A) look similar to controls not extracted before fixation (cf. Figures 1A, 2A). In contrast, microtubules of cell models decay within minutes if anti- β -tubulin is added in MSB. In the integrity index introduced in Figure 3, the microtubular patterns shown here would score as '10' (A), '3' (B) and '1' (C). (C, inset) Mitotic cell incubated in 0.2% Triton X-100 and 1 mg/ml anti- β -tubulin in MSB for 1 h, prior to fixation. Spindle fibers appear normal. Bar: 10 μ m.

Table 1. Effect of various concentrations of anti-beta tubulin on microtubules in detergent-extracted cells

Antibody in MSB ¹ (mg/ml)	Incubation time				
	1-5 min	30 min	60 min	90 min	3-12 h
0.1	3 ²	1	0	0	0
0.02	8	3	1	0	0
0.01	9	6	4	1	0
0.005	9	8	nd ³	5	nd
0.0025	9	9	9	8	8
0 (controls)	9	9	9	9	8

¹Microtubule stabilising buffer, see Materials and methods.

²These numbers refer to the arbitrary 'integrity index' of 1-10, explained in Figure 3. Each number represents the average of 30-50 cells.

³Not determined.

microtubule formation was seen after temperature shift to 37°C. Similarly, when microtubules were pre-assembled (Figure 6A), and then incubated with antibodies at 37°C, they disintegrated within minutes. In the electron microscope, only amorphous aggregates and fibers of ~10 nm diameter were visible (Figure 6B). They consisted of several subfibrils 3-4 nm wide and did not show an axial periodicity around 4 nm, in contrast to tubulin protofilaments. They were not seen when MAP-free microtubules were disrupted by the antibody. Thus, these structures probably comprise aggregated MAPs (see also Mandelkow *et al.*, 1985).

The process of antibody-induced microtubule decay could be retarded by stabilising agents, e.g., glycerol. In this case, electron micrographs taken from material fixed 10 min after the addition of the antibody revealed a complex mixture of wispy fibers either protruding from the microtubules and bundles, or, dispersed throughout the background (Figure 6C). The bundles contained irregularly associated subfibers that resembled protofilaments, as judged by their diameter (~5 nm) and axial periodicity (~4 nm). Hybrid forms (e.g., a microtubule gradually turning into a fibrous bundle) have not been observed.

Discussion

Dynamics of microtubules in tissue culture cells have been studied so far by applying physical or chemical modulators of microtubular structure, like temperature and pressure shifts, Cationophores and various drugs like colchicin and derivatives, taxol, nocodazol or vinca alkaloids (for review, see Dustin, 1984). The present study reports on a novel immunological tool: a specific polyclonal antibody with high affinity for one subunit of the heterodimeric tubulin molecule. While all other polyclonal or monoclonal anti-tubulins tested so far in microinjection experiments did not interfere with microtubular structure, this antibody severs and subsequently breaks down microtubules. This process can be clearly followed with immunofluorescence in injected cells fixed at various time points after injection.

To visualise the breakdown of microtubules, one can either quench the injected antibody molecules and use the same antibody for indirect immunofluorescence, or use other anti-tubulins reacting with different epitopes. Disassembly of microtubules in living cells was observed within 1.5 h after injecting the antibody in concentrations as low as 10 μ g/ml (Figure 4). If one considers a calculated intracellular tubulin concentration of 2-3 mg/ml, an injection volume of 10^{-7} μ l (cf. Fuchtbauer, 1984) corresponding to 5-10% of the cellular volume, one arrives at a ratio of 1 antibody molecule per ~10 000 dimeric tubulin molecules. Since the antibody should be randomly bound, single molecules can be expected to act individually along the microtubule. Ten thousand tubulin dimers, ~8 nm each and packed into the 13 microtubular protofilaments, correspond to a stretch of 6 μ m microtubular length. Thus, at 10 μ g/ml anti-tubulin in the injection solution, antibody molecules can be expected on average (provided none of them would bind to soluble tubulin dimers in the cell) every 6 μ m along the microtubules. However, even with this low antibody concentration, the microtubular pieces observed were much shorter (<1 μ m). This indicates that once microtubules are nicked by the attack of a few antibody molecules, the pieces must disintegrate spontaneously. Thus, the dis-

Fig. 6. Electron micrographs of microtubules and antibody-induced polymorphic forms. **(A)** Control microtubules repolymerised *in vitro* by incubating 2 mg/ml microtubule protein in reassembly buffer at 37°C for 20 min. Note longitudinal tubulin protofilaments, separated by ~5 nm, with 4 nm axial periodicity. **(B)** Microtubules assembled as in **(A)** and then incubated for 10 min with anti- β -tubulin (molar ratio of tubulin:antibody 1:0.5). Microtubules have completely disappeared. Fibers of ~10 nm width are seen with many kinks and with undefined protein precipitates. Note subfibrils of 3–4 nm width (arrow). **(C)** Same as in **(B)**, but with 10% glycerol in the reassembly buffer. Microtubules decay more slowly. They are seen simultaneously with fibrous bundles of irregular substructures, often tapering at their ends, and short wispy fibers in the background (arrows). Bar: 100 nm.

assembly process, once started, is self-amplified.

In the case of the pure anti-tubulin obtained by cross-absorption (see Materials and methods), an affinity of at least 10^{-8} M for 50% binding of the antigen tubulin was measured (Herrmann, 1981; Mandelkow *et al.*, 1985). In contrast, the equilibrium constant for subunit addition to microtubules is $\sim 2 \times 10^{-6}$ M (Gaskin *et al.*, 1974). Thus, a given tubulin molecule should interact 100 times more strongly with the antibody than with its neighbouring subunits. This may be the molecular basis for the disruptive efficiency of this antibody. Such a severing effect on microtubules, induced by proteins interacting with the structural subunits with high affinity, may be analogous to the clipping of actin filaments by 'severing proteins' like gelsolin (Yin and Stossel, 1979), villin (Bretscher and Weber, 1979) or actin depolymerising factor (Chaponnier *et al.*, 1985; for reviews, see Weeds, 1982; Jockusch, 1983). In both cases, very low ratios of 'severing factor' molecules to the structural subunit of the cytoskeletal structure (tubulin or actin, respectively) are sufficient for complete disintegration of such structures in living cells as well as in cell models (Figure 5, cf. also Jockusch *et al.*, 1985). The initially created longer fragments are probably unstable and disintegrate spontaneously.

Microtubules polymerised *in vitro* and subsequently disassembled by incubation with antibody give rise to fibrous structures composed of thinner subfibers (Figure 6). Since the substructure of the fibers is distinct from that of tubulin protofilaments, we sug-

gest that they consist of MAPs derived from the outer coat of microtubules. They usually show several subfibrils. In contrast, when microtubule disassembly is retarded by glycerol, thick tapered bundles with protofilament-like subfibers are observed, but direct transformation of microtubules into the bundles seems unlikely, as indicated by the absence of hybrid forms. The experiments concerning the effect of anti- β -tubulin on microtubules *in vitro* are described elsewhere in more detail (Mandelkow *et al.*, 1985). The pictures shown in Figure 6 have been obtained with antibody concentrations much higher than the one used for studies on living cells and cell models. Thus, a direct comparison of the results is not possible, and quantitation of the electron microscopic analysis was not the aim of the present study. However, we include these data in our paper to emphasize that the deleterious effect of the antibody with high affinity for β -tubulin is expressed in three different situations: in the living cell, in detergent-treated cell models and on *in vitro* assembled microtubules.

In analogy to the results obtained with many different micro-injected proteins (see Stacey and Allfrey, 1977), the injected antibody is cleared from the cytoplasm of epithelial and fibroblastic cells, and the injected cells reestablish their full complement of cytoplasmic microtubules. At high antibody concentrations, most microtubules were broken down completely, and the cells were filled with fibrous and amorphous aggregates. During the recovery period, these aggregates disappeared while new micro-

tubules seemed to grow out from the perinuclear area. Thus, while the disintegration appeared rather independent of the polarity of the intact microtubule, recovery included polar growth. With low antibody concentrations, recovery may include reannealing of microtubular pieces. Even with the highest antibody concentration used (molar ratio within the cell ~ 1 antitubulin molecule per 100 tubulin dimers), many injected cells displayed a normal microtubular pattern within 5 h after injection.

The resistance of mitotic microtubules in intact cells as well as in cell models to the anti-tubulin employed is rather surprising. The reason for this might be a difference in accessibility of the epitopes between interphase and mitotic microtubules. The epitopes recognised by the anti- β -tubulin are located in the small C-terminal domain of β tubulin (see Mandelkow *et al.*, 1985). Chemical crosslinking studies show that this small C-terminal domain of β tubulin and the large N-terminal one of α tubulin form the intradimer bond (Kirchner and Mandelkow, 1985). Possibly, in spindle fibers, this region is not as easily accessible as in individual cytoplasmic microtubules of interphase cells.

Materials and methods

Cells

The following cells were used for microinjection experiments as well as for the preparation of cell models. (i) Cell lines: rat kangaroo kidney epithelial cells (PtK₂) and human embryonic lung fibroblasts (WI 38). (ii) Primary cells: embryonic skin fibroblasts from mouse and rat, mammary duct epithelial cells from pregnant mice. Cells were seeded onto glass coverslips and grown in medium (MEM) supplemented with 7% fetal calf serum.

Cell models

To prepare detergent-extracted cell models, cells were rinsed twice for 30 s in a microtubule stabilising buffer (MSB, containing 1 mM GTP in 0.1 M morpholino ethane sulfonic acid, 1 mM EGTA, 4% polyethylene glycol 6000, pH 6.9, cf. Osborn *et al.*, 1978) and then incubated for 4 min in MSB containing 0.2% Triton X-100. Cells were rinsed twice for 30 s in MSB and incubated either in MSB or in 0.0025–0.1 mg/ml antibody in MSB for various time periods at room temperature.

Antibodies

Anti- β -tubulin was raised in specific pathogen-free rabbits at EMBL Heidelberg. Specific IgG was purified and characterised in detail (Herrmann, 1981; Mandelkow *et al.*, 1985). Briefly, α - and β -tubulin subunits were prepared by preparative gel electrophoresis and coupled to hemocyanin (KLH) to improve the immunogenicity (Ternynck and Avrameas, 1976). A 1:1 emulsion with complete Freund's adjuvant was injected directly into the popliteal lymph nodes (50 μ g per lymph node) and at multiple intradermal sites along the back. Boosters were applied at 3–6 week intervals with incomplete Freund's adjuvant. Specific IgG was obtained from several bleedings by affinity chromatography on tubulin-Sepharose 4B. α - and β -specific antibodies were obtained by heterologous cross-absorption on the corresponding affinity columns using Sepharose 4B coupled to β - or α -tubulin. Specificity and affinity of the antibodies were analysed in Western blots, indirect immunofluorescence and radioimmune assays (see Sepharose and eluted with 4 M MgCl₂ (termed ' β -cross-reacting fractions' in the paper by Mandelkow *et al.*, 1985). (ii) A subfraction of (i), obtained by passing this antibody over α -tubulin-Sepharose (' β -specific antibody'), which showed half the paper by Mandelkow *et al.*, 1985). (ii) A subfraction of (i), obtained by passing this antibody over α -tubulin-Sepharose (' β -specific antibody'), which showed half maximal binding to β -tubulin at 10^{-8} M). In living cells and cell models, no difference was found between the effect of these two antibodies.

FAB fragments of (ii) were used for studies on *in vitro* polymerised microtubules. They were obtained by papain digestion of crude serum and subsequent affinity chromatography on β -tubulin-Sepharose 4B (Mandelkow *et al.*, 1985).

The monoclonal antibody against the tyrosylated form of α -tubulin (YL 1/2) was obtained from Sera Lab. Ltd., Sussex, UK.

Microinjection

The microinjection technique introduced by Graessmann and Graessmann (1976) was used, in conjunction with an inverted Zeiss microscope and a Leitz micromanipulator (Füchtbauer, 1984; Jockusch and Füchtbauer, 1985). Glass capillaries with an opening of ~ 1 μ m were used. Pressure was applied through a 50-ml glass syringe. On average, 10^{-7} μ l was injected per cell (Füchtbauer *et al.*, 1983). On each coverslip, 50–100 cells within a marked area were injected

within 5 min. After injection, cells were placed back into the incubator for various time periods before fixation.

Indirect immunofluorescence

Cells microinjected with anti-tubulin or control proteins were fixed in -20°C cold methanol or in 4% formaldehyde followed by extraction in 0.1% Triton X-100. They were stained according to one of the two following protocols. (i) The fixed cells were incubated with goat-anti-rabbit IgG (GAR) for 30 min, washed extensively in phosphate buffered saline (PBS) and stained with anti- β -tubulin (0.1 mg/ml) followed by affinity-purified GAR coupled to lysamine rhodamine (Brantzaeg, 1973). (ii) The fixed cells were stained with the monoclonal rat antibody against α -tubulin (Kilmartin *et al.*, 1982) and affinity-purified rabbit anti-rat IgG coupled to lysamine rhodamine. Detergent-extracted cell models were fixed in 4% formaldehyde in PBS and rinsed in PBS before being incubated with anti- β -tubulin (0.1 mg/ml in PBS) and subsequently with rhodamine-coupled GAR. The stained preparations were examined in a Zeiss photomicroscope III with a 63x objective lens. Photographs were taken with automatic exposure on Tri-X Pan Kodak film.

Protein preparation and electron microscopy

Microtubule protein was prepared by three cycles of assembly and disassembly following Borisy *et al.* (1975), with minor modifications. The reassembly buffer was 0.1 M Pipes pH 6.94, 1 mM each of MgSO₄, GTP, EGTA and DTT. Tubulin was freed of MAPs by phosphocellulose chromatography according to Weingarten *et al.* (1975) with extensive modifications (for details, see Mandelkow *et al.*, 1985). Protein samples were examined by negative stain electron microscopy at concentrations ~ 1 –2 mg/ml. In order to test the reaction between microtubules and antibodies, microtubule protein of 2–4 mg/ml was polymerised in reassembly buffer at 37°C for 10 min. A 20 μ l drop of this solution was placed on a Teflon-coated metal block thermostatted at 35°C in a wet chamber and mixed with aliquots of antibody or Fab fragments of anti- β -tubulin in reassembly buffer (molar ratio tubulin:antibody typically 1:0.5 to 1:2). Incubation times were between 1 and 15 min. Controls were in buffer without antibodies. In some experiments, microtubules were stabilised by 10–25% glycerol which was also added to the antibody solution. Grids were placed on the protein drops for 5–10 s, transferred successively to six drops of 1–2% uranyl acetate or formate, excess stain was removed with Whatman filter paper nr. 1. The copper grids (600 mesh) were coated with 5–10 nm carbon films and usually glow discharged for ~ 5 s (10 Torr, 500 kV) just before use. Specimens were observed in a Phillips EM 400 T electron microscope at 100 kV, magnification 46 000 and photographed on Kodak 4489 electron microscope film.

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