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Antibody Against Tubulin: The Specific Visualization of Cytoplasmic Microtubules in Tissue Culture Cells

(microfilaments/immunofluorescence/colchicine/cell structure)

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ABSTRACT **Cytoplasmic microtubules in tissue culture cells can be directly visualized by immunofluorescence microscopy. Antibody against tubulin from the outer doublets of sea urchin sperm flagella decorates a network of fine cytoplasmic fibers in a variety of cell lines of human, monkey, rat, mouse, and chicken origin. These fibers are separate and of uniform thickness and are seen throughout the cytoplasm. The fibers disappear either in a medium containing colchicine or after subjection of the cells to low temperature. The same treatments do not destroy the microfilamentous structures that are visualized by means of antibody against actin. When trypsin-treated enucleated cells are replated and then stained with antibody against tubulin, the fibers can be seen to traverse the entire enucleated cytoplasm.**

Electron microscopy of cells grown in culture has shown that at least three major fibrous structures can be found in a wide variety of cells. These are microfilaments (6 nm diameter), filaments (10 nm), and microtubules (25 nm). It is generally assumed that these fibrous systems are responsible for the different types of movement of the eukaryotic cell: movement of the individual cell, changes in shape of the cell, and movement of organelles within the cell (see, for example, refs. 1–4).

Recently antibodies against actin have been used to show by indirect immunofluorescence that cultured cells contain an elaborate network of actin-containing fibers (5). These actin-containing fibers correlate well with the microfilament system (6–8), and immunofluorescence studies with antibodies against smooth muscle myosin (9) have shown that this structural protein also is localized in or very close to the microfilament system.

In an attempt to visualize microtubular structures in tissue culture cells by the same technique, we have used an antiserum directed against tubulin from outer doublets of sea urchin sperm. Since this tubulin has been shown previously to be very similar in amino-acid sequence to tubulin from chicken brain (10), it was hoped that the antiserum against sea urchin tubulin would show immunological cross-reaction with tubulin from chicken and mammalian cells. Here we report that the antibody decorates a network of fine cytoplasmic fibers in a variety of cells of human, monkey, rat, mouse, and chicken origin. The *pattern* of fibers is distinct from those reported previously for actin and myosin (5, 9). The fibers that can be decorated with antibodies against tubulin disappear when cells are exposed to colchicine or to low temperature, whereas the microfilament fibers do not.

Abbreviation: PBS, phosphate-buffered saline.

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MATERIALS AND METHODS

Cells. 3T3, an established cell line of mouse embryonic origin (11), was grown in Dulbecco's modified Eagle's medium with 10% calf serum. Secondary chick embryo fibroblasts were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Growth of monkey BSC-1 cells, enucleation of these cells after cytochalasin B treatment, and replating of enucleates were performed as described previously (12).

Colchicine sensitivity of microtubules was assayed by the following experiment: Mouse 3T3 cells were trypsinized and allowed to attach to glass coverslips in a medium containing the drug at 15 μg/ml. They were kept for 20 hr at 37° before processing for immunofluorescence.

Cold sensitivity of microtubules was assayed on 3T3 cells grown on glass coverslips by the following procedure: The cells were made permeable to macromolecules by Ishikawa's procedure (13), i.e., cells were treated at 4° with a series of solutions decreasing in glycerol concentration from 50% to 5%. Since glycerol is known to stabilize microtubules (14), the coverslips were treated at 4° with Ishikawa's buffer (13) without glycerol. They were left in this buffer for 12 hr with one buffer change in order to remove the glycerol and to subject the microtubules to low temperature, which is known to depolymerize these structures *in vivo* and *in vitro* (15–17). The coverslips were then processed for immunofluorescence with and without fixation with formaldehyde (see below). No difference in the results was obtained.

Preparation of Antiserum to Microtubule Protein. Outer doublet microtubules of sperm flagella from sea urchins (*Strongylocentrotus purpuratus*) prepared according to Stephens *et al.* (18) were used as antigen. The antiserum used in the following studies was obtained by pooling two individual antisera having identical properties, but prepared under somewhat different immunization conditions. Schedule 1: 50 mg of doublets, homogenized in phosphate-buffered saline (PBS) with Freund's complete adjuvant (Difco) were injected intramuscularly into a rabbit at weeks 0, 4, and 10; 5 mg more were injected subcutaneously at week 12. The rabbit was bled 5 days thereafter, and 5 days after subsequent subcutaneous injections. Properties of this antiserum have al-

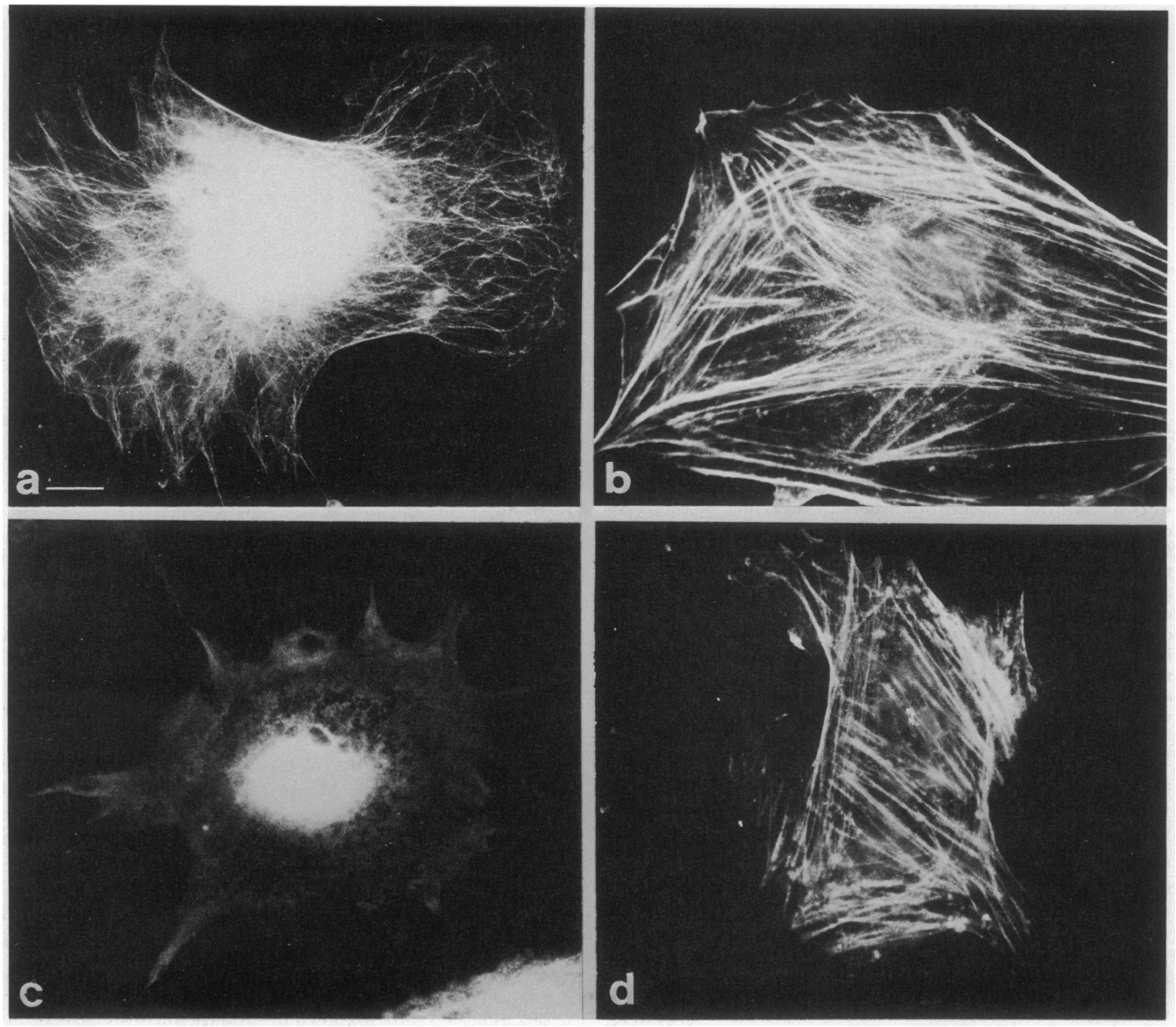

FIG. 1. Indirect immunofluorescence using antibodies against tubulin and actin. (a) 3T3 cells stained with tubulin antibody, (b) 3T3 cells stained with actin antibody, (c) as (a) but after colchicine treatment, (d) as (b) but after colchicine treatment. Note disappearance of fine cytoplasmic tubulin-containing fibers after colchicine treatment. Nuclear fluorescence seen here is not seen in cells of other species treated similarly (see *Discussion*). The bar indicates 10 μm.

ready been described (19). Schedule 2: The antigen consisted of a mixture of unfixed and glutaraldehyde-fixed (2 hr at 4° in 3% glutaraldehyde, 50 mM Tris·HCl, pH 7.0) doublet microtubules homogenized in PBS with Freund's complete adjuvant. Fifteen milligrams of antigen were injected intramuscularly, followed in 5 months by three monthly subcutaneous injections of 3 mg. Blood was collected 5 days thereafter.

Two tests were used to demonstrate that the serum contained tubulin-specific antibodies. First, the serum was tested by immunodiffusion in agarose with two different preparations of tubulin: flagellar outer doublet microtubules obtained by mercurial extraction, and isolated mitotic apparatus obtained by mercurial extraction and 0.6 M KCl extraction (for procedures see ref. 19). The single precipitin line obtained with the second tubulin preparation showed identity with the line given with the first tubulin preparation. A faint second precipitin line arising later in the assay with sperm tail preparations probably represents a sperm-specific protein. Furthermore, the major band obtained with sperm tail tubulin was confluent (with a spur) with the band given by the anti-tubulin serum described by Fulton *et al.* (20), a sample of which was generously supplied by Dr. Fulton. Since this latter antiserum has been shown previously to decorate microtubules in electron microscopic studies (20), we conclude that our antiserum has tubulin-specific antibodies. The second test for tubulin-specific antibodies involved the use of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and urea to analyze the immune precipitate obtained by mixing the γ-globulin fraction of our antiserum with a mercurial extract of sperm flagellar outer doublet microtubules. After electrophoresis and staining of the gel, a

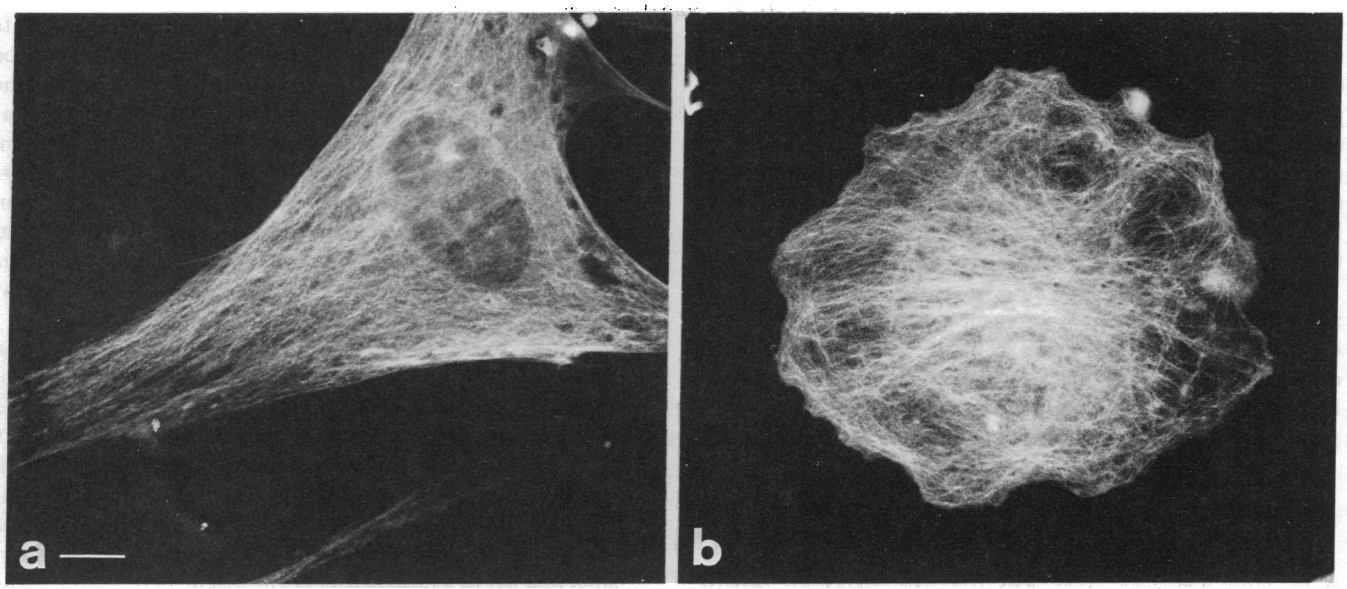

FIG. 2. Indirect immunofluorescence using tubulin antibody. (a) Chick embryo fibroblast, (b) BSC-1 enucleate after trypsinization and replating. The bar indicates 10 μm.

strong band migrating with the same mobility as tubulin was observed.

The γ-globulin fraction of the antiserum was purified by three precipitations with 14% sodium sulfate, desalting on Sephadex G-25, and chromatography on DEAE-cellulose (21). The γ-globulins were stored at 15 mg/ml in PBS. For immunofluorescence studies the γ-globulins were diluted 1:15 into PBS.

Immunofluorescence. Cells were grown on glass coverslips. For fixation the medium was removed and the cells were directly treated with 3.5% formaldehyde in PBS for 5 min at room temperature. Coverslips were washed quickly with PBS and then treated with methanol at $-20°$ for 4 min followed by acetone at $-20°$ for 2 min. After air drying the coverslips were processed essentially as described previously (5, 9). The fluorescein-labeled goat antibodies to rabbit γ-globulins (Miles Co.) were diluted 1:10 into PBS. The coverslips were viewed with a Zeiss microscope equipped with epifluorescent optics. Pictures were taken with an oil immersion objective (63×) on Kodak Plus X film.

The rabbit antibody to mouse actin has been described previously (5).

RESULTS

Cells grown on glass coverslips were fixed in formaldehyde for optimal preservation of fibrous material and then processed for indirect immunofluorescence as described in *Materials and Methods*. Figs. 1a and 2a show typical fluorescent patterns obtained with anti-microtubule antibody for mouse 3T3 cells (Fig. 1a) and chick embryo fibroblasts (Fig. 2a). Both cells display a multitude of very thin, separate fluorescent fibers of apparently constant diameter within the cytoplasm. These fibers traverse the whole cytoplasm and only a few fibers are arranged parallel to the margins of the cell. Some of the fibers are straight and some are bent or curved (Figs. 1a and 2a). The intense nuclear staining seen in mouse 3T3 cells (Fig. 1a) but not with chick embryo cells (Fig. 2a) is discussed below (see *Discussion*).

Extensive electron microscopic studies have proven that cytoplasmic microtubules disappear in the presence of colchicine (see, for instance, refs. 3 and 4). Therefore, mouse 3T3 cells were treated with colchicine as described in *Materials and Methods*. Although somewhat reduced in size as a consequence of colchicine treatment, the cells were well spread over the surface of the coverslip. After processing for immunofluorescence, no organized fluorescent fibers appeared in the cytoplasm when antibody against tubulin was applied (Fig. 1c). Instead, there was a diffuse distribution of weak fluorescence within the cytoplasm. The intense fluorescence within the nucleus resembled that seen in those cells in the absence of colchicine (Fig. 1a). In the absence of substructure it is not possible to determine whether this nuclear fluorescence (Fig. 1c) is tubulin-specific or whether it is different from the fluorescence seen in nuclei of untreated 3T3 cells (Fig. 1a).

The expression of microtubules in the cytoplasm was also studied in normal and enucleated monkey BSC-1 cells. The pattern of cytoplasmic fibers decorated by the antibodies in BSC-1 cells was similar to the one seen in mouse 3T3 cells (Fig. 1a). When BSC-1 cells were enucleated and the enucleates were trypsinized and replated, they showed an extensive pattern of fragile cytoplasmic fibers. Given sufficient time to allow full spreading of the enucleates (8–12 hr), the cytoplasmic fibers crossed the entire enucleated cytoplasm (Fig. 2b). Enucleated BSC-1 cells were unable to assemble stainable cytoplasmic fibers after replating in the presence of colchicine (data not shown).

The pattern of organization of actin in a variety of cells of different origin has been described previously in detail (5, 6, 8). In order to distinguish between microtubular fibers and actin-containing fibers, both normal and enucleated cells were processed in parallel for staining with tubulin antibody and actin antibody. Fig. 1b shows a 3T3 cell stained with actin antibody as described originally by Lazarides and Weber (5). The actin and microtubule fibers in the cytoplasm are remarkably different in appearance and in arrangement. The actin fibers (5) differ in diameter (up to 1 μm) and length, are concentrated toward the adhesive side of the cell, and are arranged predominantly parallel to each other. In contrast,

the microtubular fibers are separate from each other, are very thin, and show no strong orientation along the boundary of the cell. The two fibrous systems are further distinguished by replating 3T3 cells into a medium containing colchicine. Cells treated this way display actin fibers (Fig. 1d) but not cytoplasmic microtubules (Fig. 1c). A third difference between these two fibrous systems can be observed in cells that have been made permeable by glycerination and have then been treated in the cold with buffer not containing glycerol (see *Materials and Methods*). When cells treated by this procedure are subjected to immunofluorescence, actin-containing fibers can readily be seen, whereas cytoplasmic microtubular fibers are absent (data not shown). This result conforms with the known property of microtubules to depolymerize at low temperatures both *in vivo* and *in vitro* (14–17).

DISCUSSION

We have shown by indirect immunofluorescence that antibody against tubulin prepared from sea urchin outer doublets decorates a delicate system of fibers in the cytoplasm of tissue culture cells. Mouse 3T3, monkey BSC-1 cells, and normal secondary fibroblasts of human, hamster, rat, and chicken origin have cytoplasmic fibers showing similar structural organizations and patterns of distribution. This uniformity of a cytoplasmic component in cells of widely different origin may be explained by the conservative amino acid sequence of the tubulin protein (10). Although we do not yet know if the antiserum against tubulin used in this study is a precipitating antiserum for tubulin of mammalian cells, we do know that the antiserum does precipitate the original antigen, tubulin from sea urchin outer doublets (see *Materials and Methods*), and that this tubulin and vertebrate tubulins are quite similar in amino acid sequence and structure (10). Since the antiserum is known not to be monospecific (see *Materials and Methods*), we cannot exclude the possibility that our antibody preparation contains antibodies directed against proteins other than microtubules. However, we have shown that the cytoplasmic fibers decorated by the antibody preparation are sensitive to colchicine and low temperature, sensitivities which have been found in extensive electron microscopic studies to be specific for microtubules (see, for example, refs. 3, 4, and 14–17). In addition, preliminary results show that the antibody preparation specifically stains the spindle in mitotic mammalian cells (Weber, unpublished), again suggesting that the antibody preparation is capable of specific interaction with mammalian tubulin.

The nuclear staining found in 3T3 cells could be due at least in part to tubulin present in these organelles. However, the observed nuclear fluorescence is most likely not tubulin-specific. It appeared only in nuclei of 3T3 cells. It did not appear in nuclei of chick embryo fibroblasts (Fig. 2a) or in nuclei of cells of the PtK_1 line of rat-kangaroo. Therefore, we assume that the rabbit antiserum recognizes in some cell lines, notably 3T3, an antigen not related to tubulin, which gives rise to strong nuclear fluorescence. Final proof for this interpretation can be expected after isolation of tubulin-specific γ-globulins out of the pool of total rabbit γ-globulins.

It is difficult to evaluate the significance of the diameter of the microtubular fibers revealed by immunofluorescence. Electron microscopic measurements of microtubules (see, for example, refs. 1, 3, and 4) show them to be 25 nm in diameter, far below the 200 nm limiting resolution of the light microscope. However, darkfield ultraviolet epi-illumination permits a fluorescent fiber of any thickness less than 200 nm to be visualized at this limit of resolution, due to the fact that the fiber is emitting, rather than absorbing, light. Therefore, at this resolution it is impossible to distinguish between individual microtubules and bundles of up to 10 microtubules by direct measurement of the diameters of the fluorescent fibers. However, since electron microscopic studies show that lateral bundles of microtubules are rarely found in the cytoplasm of tissue culture cells, we prefer the assumption that we are visualizing individual microtubules.

This is the first time to our knowledge that intracellular cytoplasmic microtubules have been visualized by immunofluorescence microscopy. A previous report (22) showed that antibodies against vinblastine-induced paracrystals [which are assumed to be pure tubulin (23)] decorate mitotic figures in mitotic cells of different species (22, 24). These studies did not reveal cytoplasmic fibers in nonmitotic cells, probably because the fixation procedure used was rather harsh and led to a destruction of the fragile cytoplasmic structures (Weber, unpublished results).

Several lines of evidence indicate that the cytoplasmic microtubular fibers are distinct from the microfilament fibers, which contain actin (5) and myosin (9). The display of the microtubular fibers, as well as their uniform diameter and their sensitivity to colchicine and low temperature, clearly distinguishes them from the actin-containing fibers. Although the microtubule and microfilament fibers appear to differ in their displays, the possibility remains that these two fibrous systems may be connected in some yet unknown way.

Finally, since the conditions for immunofluorescent visualization of the microtubular system in tissue culture cells are now known, it should be possible to determine by a similar approach whether other proteins of the cell form part of the microtubular system or are connected to it.

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