

ELECTRON MICROSCOPE DEMONSTRATION OF TUBULIN IN CILIA AND BASAL BODIES OF RAT TRACHEAL EPITHELIUM BY THE USE OF AN ANTITUBULIN ANTIBODY

RONALD E. GORDON, BERNARD P. LANE, and FREDERICK MILLER

From the Department of Pathology, Health Sciences Center, State University of New York, Stony Brook, New York 11794

ABSTRACT

It has been previously demonstrated that both cytoplasmic microtubules and the microtubules of cilia, flagella, and sperm tail contain tubulin. Although the morphology of cytoplasmic microtubules and that of axonemes differs in cells from which they have been isolated, the tubulin of the two structures shares physical and chemical properties. In some mammalian tissues, such as tracheal epithelium, cilia and basal bodies are difficult to isolate and characterize. The use of an enzyme-labeled immunoglobulin probe would facilitate identification and *in situ* localization of such proteins.

Tubulin prepared from porcine brain by ion-exchange chromatography and from rat brain by the method of cyclic polymerization and depolymerization (5, 14) with subsequent disk gel electrophoresis with SDS (17) were injected intravenously into rabbits. The animals were intermittently bled and the antisera extracted. The specificity of the antisera was proved by indirect immunofluorescence staining of the mitotic spindle, specific blocking of spindle staining by purified tubulin and not by other proteins, staining of 3T3 cytoplasmic microtubules, single line on immunoelectrophoresis, failure of control antisera to show any of these, and precipitation of antibody with all tubulin preparations and not with actin.

We have shown by electron microscopy of ciliated cells of the tracheal epithelium stained with antitubulin by the indirect enzyme-labeled antibody method that the basal bodies, outer doublets, and central pair of the cilia contain tubulin. This indicates that tubulin in microtubules of cilia and basal bodies of rat tracheal epithelium is antigenically similar to tubulin extracted from cytoplasmic neurotubules of brains from the same species and from a different mammalian species. No other axonemal structures stained with the antitubulin. Three different preparations of tubulin from pigs and rats were used to immunize rabbits. All elicited similar antisera which gave identical staining patterns. The specificity of the staining was demonstrated by the absence of staining with immune serum absorbed with purified tubulin, the absence of staining with preimmune serum, and the absence of staining if any of the reagents were omitted during the staining reaction.

KEY WORDS tracheal epithelium · cilia · tubulin · immunoperoxidase · antisera · electron microscopy

It has been shown biochemically that microtubules of cells (11) and axonemes of flagella from protozoans (8) are composed of tubulin, and immunofluorescence techniques have been used to demonstrate that cytoplasmic microtubules (2) and the mitotic spindle (5) of mammalian cells contain this protein. Tubulin has also been recognized as a constituent of neurotubules of brain cells by immunocytochemistry at the ultrastructural level (7). The morphology, molecular constituents, and organization of tubules which comprise the axonemes of cilia, flagella, sperm tail, and basal bodies have been extensively studied and are discussed in detail in three recent reviews (4, 6, 15). The morphology of cilia and basal bodies of the respiratory tract epithelium has been studied (13, 16), but the molecular constituents forming these structures have not been determined. We have applied a modification of the highly sensitive enzyme-labeled anti-immunoglobulin technique of Nakane and Pierce (10) to tracheal epithelium of the rat to determine whether axonemes and basal bodies of ciliated cells contain proteins antigenically similar to tubulin derived from brain.

MATERIALS AND METHODS

Preparation of Antigen

Two sources of tubulin were employed without any detectable difference in the resulting antisera. The first, which was a generous gift from Doctors G. Borisy and D. Murphy of the University of Wisconsin, had been prepared from porcine brain by ion-exchange chromatography. The second was obtained from rat brain by using the method of cyclic polymerization and depolymerization described by Fuller et al. (5) and Shelanski et al. (14) or by the use of subsequent preparative disk gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) as described by Wiche and Cole (17). The first preparation was judged to be 95% pure and the latter over 99% pure by SDS-gel electrophoresis.

The SDS gels of the crude and final tubulin preparations are shown in Fig. 1. The gels were prepared and run according to the method of Porzio and Pearson (12).

Preparation of Antisera

Antisera were prepared as previously described (9). 0.2 mg of tubulin was mixed with a slurry of diethylaminoethyl (DEAE)-Sephadex-A50 (1 ml) and cross-linked with glutaraldehyde. An emulsion was prepared with Freund's complete adjuvant (1:1) and administered subcutaneously to rabbits. After two doses at weekly

intervals, animals received the tubulin-DEAE slurry as a dilute intravenous suspension in 0.15 M NaCl containing 0.05 M pH 7.4 sodium phosphate, 0.5 ml containing 0.2 mg of antigen, on three occasions 10 days apart. Each animal received a total of 1.0 mg of antigen. Two to five weekly bleedings were pooled for each animal. Porcine brain tubulin was given to two animals while rat brain tubulin was administered to six animals (of which four responded, two with the Fuller preparation and two with the highly purified Wiche and Cole material). Preimmunization sera were employed as the normal control in all cases. The specificity of the antisera was determined by all of the following: ability to stain the mitotic spindle as determined by indirect immunofluorescence; specific blocking of the spindle staining by purified tubulin and not by actin, α -actinin, myosin, troponin-tropomyosin; ability of the antisera to stain 3T3 cytoplasmic microtubules (courtesy of Dr. E. Lazarides, Cold Spring Harbor Laboratories); demonstration of a single line on immunoelectrophoresis with rat brain extract and purified tubulin (Fig. 2); and failure of the control sera to show any of these results. Antisera were analyzed by the quantitative precipitin technique and were found to contain $30 \pm 10 \mu\text{g}$ antibody/ml (9).

To further assure the purity of the tubulin and the antiserum directed against it, precipitin curves were constructed by using both pure tubulins (porcine and rat brain), rat brain homogenate (adjusted to equivalent tubulin concentration based on tubulin content determined by SDS-gel analysis), and the unrelated antigen, rat skeletal muscle actin (Fig. 3). As can be seen, allowing for experimental error, the three fractions containing tubulin give superimposable curves with a single equivalence point. Actin did not produce any measurable precipitate. Furthermore, analysis of the washed precipitate at equivalence revealed only a single species (aside from the immunoglobulin) and it had a mobility on SDS-acrylamide gel identical to that of tubulin. It should also be noted that the Wiche and Cole procedure assures that the isolated material also fulfills the polymerization behavior characteristic of tubulin (17).

The serum fraction containing the antitubulin antibody of each pool was prepared by precipitation with ammonium sulfate (to a final concentration of 1.6 M). Precipitates were dialyzed extensively against saline, adjusted to 5- to 10-fold concentrations of antibody as compared to the original sera, aliquoted, and stored at -40°C until used.

After removal from rats, tracheas were fixed and immersed in an aqueous solution of 2% paraformaldehyde with 0.037 M sodium phosphate buffer adjusted to pH 7.2. After 1 h of fixation, the tracheas were split longitudinally and washed for 12 h in phosphate-buffered saline (PBS; Difco Laboratories, Detroit, Mich.) to which 10% sucrose had been added.

The washed tissues were frozen by immersion in liquid nitrogen and then thawed as a means of mildly disrupting the cells so that antisera and solutions could penetrate them. The half-tracheas were transferred to a 1:40 PBS

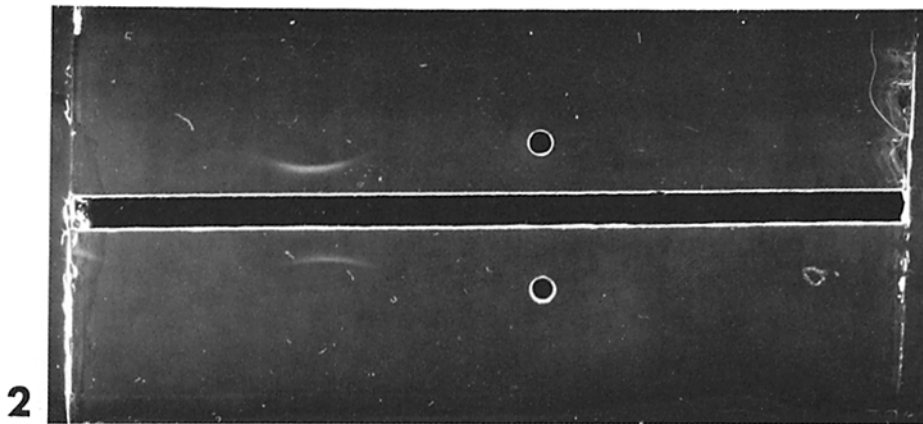
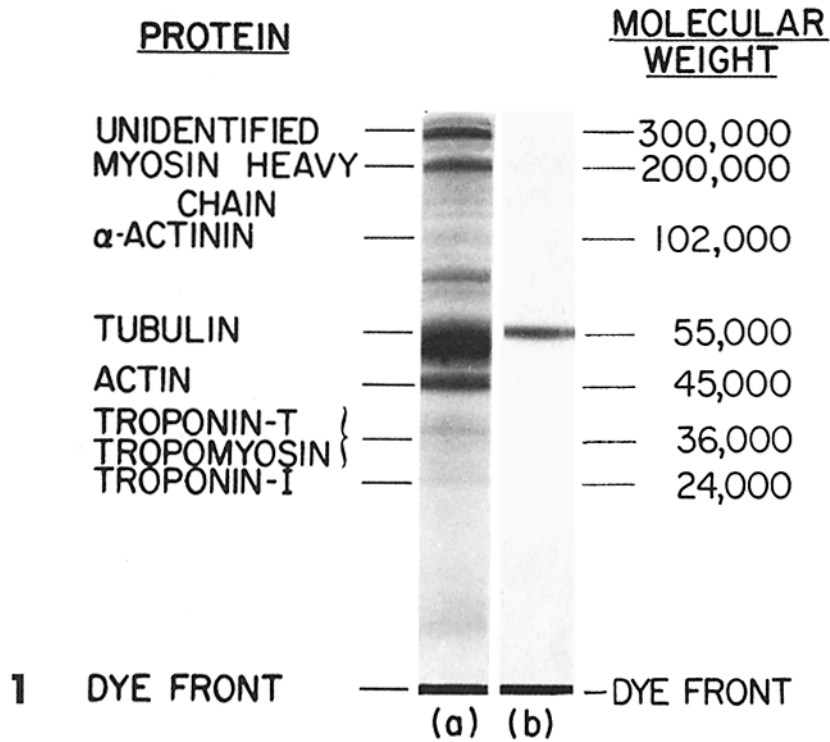


FIGURE 1 SDS-polyacrylamide gel of preparation used in this study. Gels consist of 1:100 bisacrylamide (10%), 0.4 M, pH 8.8 Tris-glycine, 5% glycerol and 0.1% SDS. In each gel there is 0.03–1.0 mg of protein (0.05 ml sample applied). Gels were run at constant current for approximately 3 h, fixed, stained with Coomassie Brilliant Blue, and destained in methanol:acetic acid:water (1:2:17). (a) Crude preparation in the isolation of tubulin from rat brain. (b) Purified rat brain tubulin prepared by cyclic polymerization and depolymerization.

FIGURE 2 Immunoelectrophoresis of crude rat brain extract (upper well) and the purified tubulin (Fig. 1 b) derived from it. Rabbit antitubulin employed in these studies is in the trough. The purified tubulin is approximately 0.1 mg/ml, the crude extract 10.0 mg/ml, and the antisera has been concentrated 10-fold with regard to the globulin fraction by precipitation with 1.6 M $(\text{NH}_4)_2\text{SO}_4$ and redissolved in PBS. Matching single lines are seen in both cases. The anode is at the left (+).

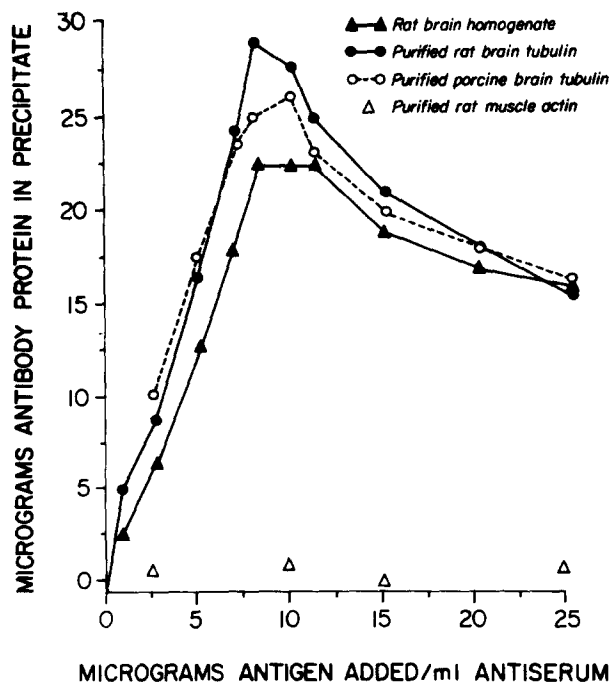


FIGURE 3 Microprecipitin analysis of rabbit antirat brain tubulin with several antigens. Each point represents the mean of duplicate determinations employing 2.5 ml of antiserum rat brain homogenate, purified rat brain tubulin, purified porcine brain tubulin, and purified rat muscle actin. No other precipitate peaks were detected when as much as 1.5 mg of tubulin was employed per milliliter of antiserum.

dilution of the rabbit antiporcine or rat tubulin (9 $\mu\text{g/ml}$ antibody final concentration) and incubated at 37°C for 1 h. Specimens were removed from the diluted antiserum and washed for 30 min in three changes of PBS sucrose. The specimens were then transferred to a 1:40 dilution of sheep Fab antirabbit IgG (SARG; 1.25 $\mu\text{g/ml}$) coupled to horseradish peroxidase (glutaraldehyde; Pasteur Institute, Garches, France) and incubated for 1 h at 37°C. After three 10-min washes in PBS-10% sucrose, the tissues were treated with 3% glutaraldehyde in PBS-10% sucrose for 30 min to fix the antibodies to the tissue sites. The fixative was thoroughly washed out with three changes of PBS-sucrose for periods in excess of 1 h. The tissues were then incubated for 7–10 min in a 0.5% solution of 3,3-diaminobenzidine tetrahydrochloride (DAB) to which H_2O_2 had been added to make a final concentration of 0.01% treated with 1% unbuffered osmium tetroxide to render the DAB reaction product electron dense, dehydrated with ethanol followed by propylene oxide, and embedded in Epon 812.

Control specimens consisted of: (a) tracheas incubated with normal rabbit serum (NRS) rather than specific antiserum against tubulin and then sequentially treated with SARG and DAB; (b) tracheas treated with SARG followed by DAB; (c) tracheas treated with DAB alone; and (d) tracheas treated with antitubulin

antiserum which had been absorbed with one of four different amounts of purified tubulin immunogen (1, 3, 5, 10 $\mu\text{g/ml}$ antiserum) and then diluted to 1:40. After incubation with this absorbed primary antiserum, the tissue was sequentially treated with SARG and DAB. The last control tests for the specificity of this antiserum when used in a histochemical staining procedure for transmission electron microscopy.

Sections, 1 μm thick, were cut from all blocks and examined with a light microscope. The presence and distribution of dense precipitates in ciliated cells were evaluated under double-blind conditions. Representative areas of labeled cells were sectioned for electron microscopy, as were random areas from blocks in which no precipitate was discerned with the light microscope. The sections were examined without staining with salts of heavy metals.

RESULTS

Cell structure was maintained in both experimental and control tissues. In particular, basal bodies, terminal filament webs, and cilia showed normal morphology. The plasma membranes, nuclei, and mitochondria were also grossly intact but exhibited focal disruption. Cytoplasmic microtubules and endoplasmic reticulum were not preserved.

A localized dense precipitate was seen in all specimens exposed sequentially to rabbit antitubulin, SARG coupled to horseradish peroxidase, and H_2O_2 -diaminobenzidine before osmium tetroxide fixation. Light microscopy revealed deposition primarily at the apical ends of ciliated cells (Fig. 4). In the controls, no precipitation was observed (Fig. 5). At the ultrastructural level, a heavy, finely particulate precipitate was localized to the outer doublet and central pair of axonemal microtubules and outer triplet microtubules of the basal bodies (Figs. 6, 8, 9). The precipitate coated the outer surface of the tubules and did not extend onto the connecting arms or the spokes of the cilia, the striated rootlets of basal bodies, or the interbasal body filaments. Controls (a-c) were free of fine precipitate at these sites (Fig. 7). All the immunogen-absorbed antisera, including the preparation treated with the lowest concentration of tubulin, were found not to produce staining (Fig. 10).

DISCUSSION

The tubules that constitute the axonemes of cilia, flagella, and sperm tails are morphologically similar to cytoplasmic microtubules. However, the arrangement ($9 \times 2 + 2$) of the tubules suggests that structural and chemical differences may exist between these tubules and cytoplasmic microtubules. Biochemical isolation of cytoplasmic micro-

tubules of neurons reveals two species of tubulin (14). Similar biochemical studies of axonemal tubules of flagella also exhibit two species of tubulin, one of which shares physical and chemical properties with one of the tubulins from neurotubules (17). Tubulin from chick brain has also been shown to assemble onto flagella and sperm tail microtubules (1). We have now demonstrated that tubulin extracted from porcine or rat brain cross-reacts antigenically with components of axonemes of cilia in the tracheal epithelium of the rat.

Although this evidence does not establish the identity of the proteins of microtubules from different cells or even prove complete chemical identity of proteins with shared antigenic sites, it does reveal a measure of similarity between microtubules from brain cells and those of cilia of cells of the respiratory tract. This degree of conservation of structure of the tubulin molecule from cell to cell across species lines has been previously reported (5, 9, 17). The difficulty in isolating purified cilia from this epithelium, which is composed of several different cell types in varying proportions, may preclude more direct tests of their composition. To an even greater extent, the limitations of isolation techniques would impede analysis of basal bodies from the ciliated cells. Electron microscopy with enzyme-labeled immunoglobulins, which permits an approach to the composition of these cell substructures *in situ*, enabled us to dem-

FIGURE 4 A $1 \mu\text{m}$ -thick section of tracheal epithelium treated first with rabbit antitubulin and then with FAB fragments of SARG coupled to horseradish peroxidase. This light micrograph exhibits the dense precipitate on the apical surface end of a ciliated cell (arrow). The dense precipitate is the osmiophilic reaction product formed after incubation of the tissue with H_2O_2 and DAB. Stained with methylene blue and azure II. Bar, $10 \mu\text{m}$. $\times 1,300$.

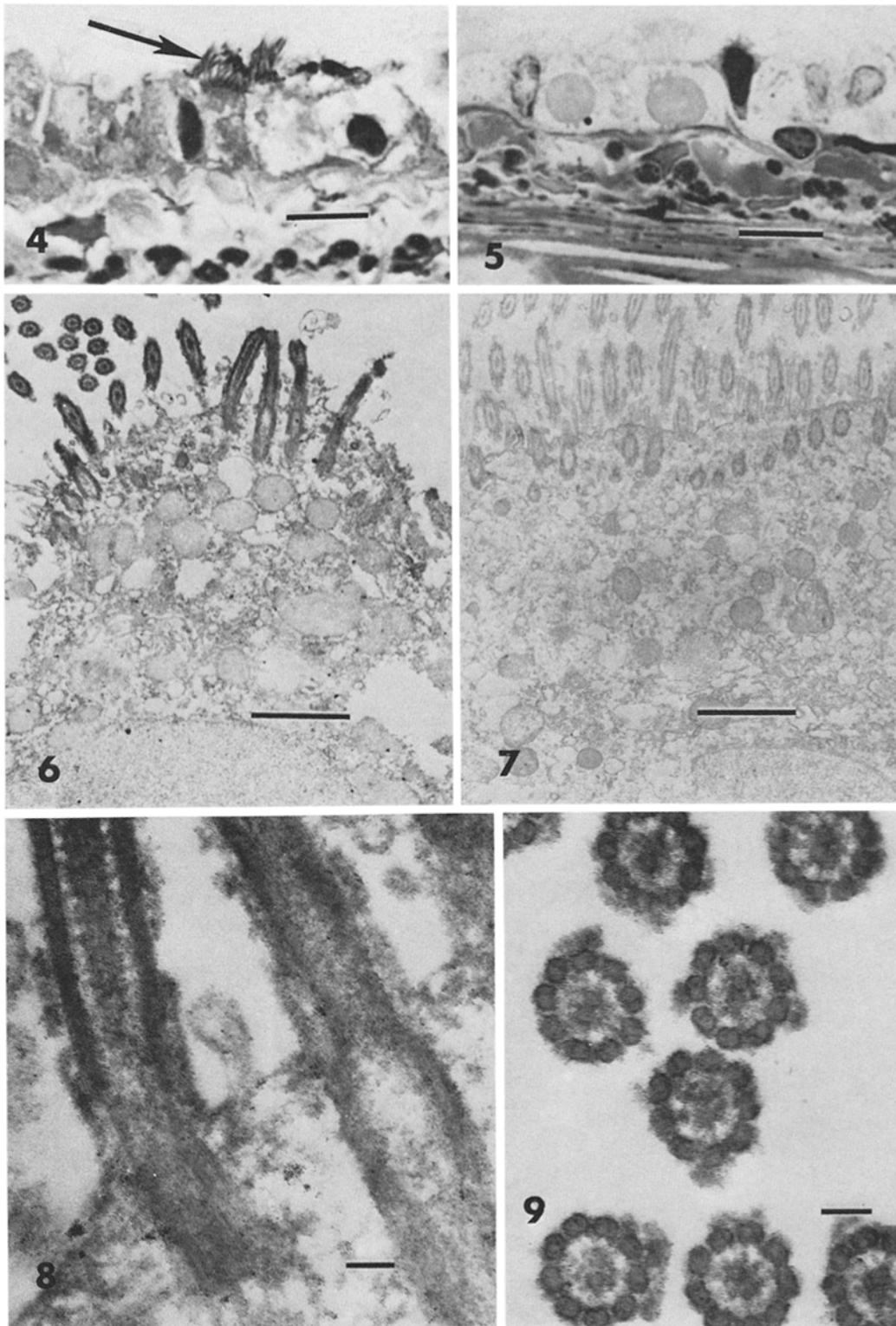
FIGURE 5 A $1 \mu\text{m}$ -thick section of control tracheal epithelium, treated with rabbit serum rather than rabbit antitubulin, does not exhibit any precipitated reaction product at the apical end and cilia of ciliated cells. Stained with methylene blue and azure II. Bar, $10 \mu\text{m}$. $\times 1,300$.

FIGURE 6 Electron micrograph of an unstained ultrathin section from tracheal epithelium treated with rabbit antitubulin and then with FAB fragments of SARG coupled to horseradish peroxidase show that the label is localized to basal bodies and axonemes of cilia. Bar, $1 \mu\text{m}$. $\times 15,000$.

FIGURE 7 Cells of control tracheal epithelium exposed to normal rabbit serum followed by horseradish peroxidase coupled FAB fragments of SARG do not show dense precipitates on ciliary tubules or basal bodies after H_2O_2 -DAB incubation and treatment with osmium tetroxide solution. Bar, $1 \mu\text{m}$. $\times 15,000$.

FIGURE 8 A longitudinal section through the cilia and basal bodies at higher magnification demonstrates antitubulin antibody localized along the tubules of the axonemes and on basal bodies. The sections are not stained with lead or uranyl salts. Bar, 200 nm . $\times 60,700$.

FIGURE 9 A cross section through cilia at higher magnification reveals sites of binding of antitubulin antibody to the periphery of the tubules of cilia. The sections are not stained with lead or uranyl salts. $\times 60,700$.



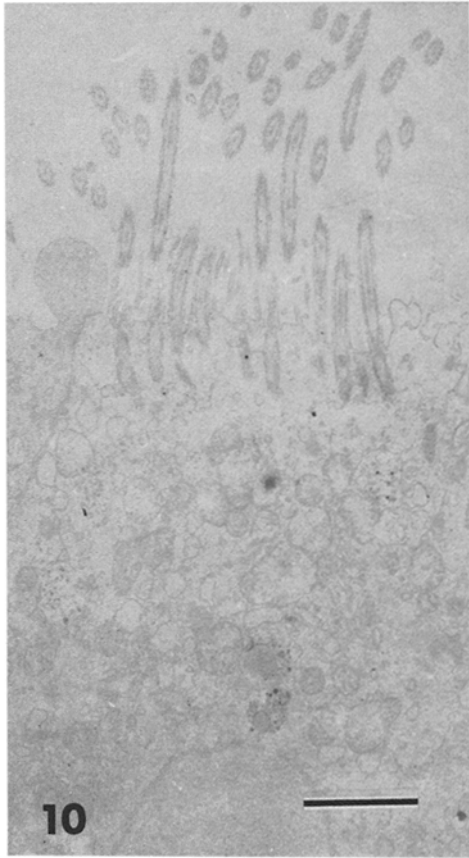


FIGURE 10 Electron micrograph of an unstained thin section from tracheal epithelium treated with antitubulin antiserum that had been absorbed with tubulin at a concentration of $1 \mu\text{g}$ tubulin/ml antiserum. The tissue was then sequentially incubated with FAB fragments of SARG coupled to horseradish peroxidase and H_2O_2 -DAB. There are no precipitates associated with basal bodies and axonemes of cilia. Bar, $1 \mu\text{m}$. $\times 15,000$.

onstrate that basal bodies also bind the antiserum with specificity for tubulin.

We would like to thank Mr. Jules Elias and Mr. Edward Drummond for their technical assistance.

This work was supported by contract N01 CP 33361 from the National Cancer Institute.

Received for publication 17 November 1976, and in revised form 22 June 1977.

REFERENCES

- BINDER, L. I., W. L. DENTLER, and J. L. ROSENBAUM. 1975. Assembly of chick brain tubulin onto flagellar microtubules from chlamydomonas and sea urchin sperm. *Proc. Natl. Acad. Sci. U. S. A.* **72**:1122-1126.
- BRINKLEY, B. R., G. M. FULLER, and D. P. HIGHFIELD. 1975. Cytoplasmic microtubules in normal and transformed cells in culture: analysis by tubulin antibody immunofluorescence. *Proc. Natl. Acad. Sci. U. S. A.* **72**:4981-4985.
- BRYAN, J., and L. WILSON. 1971. Are cytoplasmic microtubules heteropolymers? *Proc. Natl. Acad. Sci. U. S. A.* **68**:1762-1766.
- DUSTIN, P. 1974. Some recent advances in the study of microtubules and microtubule poisons. *Arch. Biol.* **85**:263-288.
- FULLER, G. M., B. R. BRINKLEY, and J. M. BOUGHTER. 1975. Immunofluorescence of mitotic spindles by using monospecific antibody against bovine brain tubulin. *Science (Wash. D. C.)* **187**:948-950.
- INOUE, S., and R. E. STEPHENS, editors. 1975. *Molecules and Cell Movement*. Raven Press, New York. 143-207.
- MATUS, I. A., B. B. WALTERS, and S. MAGHAL. Immunohistochemical demonstration of tubulin associated with microtubules and synaptic junctions in mammalian brain. *J. Neurocytol.* **4**:733-744.
- MEZA, I., B. HUANG, and S. BRYAN. 1972. Chemical heterogeneity of protofilaments forming the outer doublets from sea urchin flagella. *Exp. Cell Res.* **74**:535-540.
- MILLER, F., E. LAZARIDES, and J. ELIAS. 1976. Application of immunologic probes for contractile proteins to tissue sections. *Clin. Immunol. Immunopathol.* **5**:416-428.
- NAKANE, P. K., and G. B. PIERCE. 1967. Enzyme-labeled antibodies for the light and electron microscopic localization of tissue antigens. *J. Cell Biol.* **33**:307-318.
- OLMSTED, J. B., G. B. WITMAN, K. CARLSON, and J. L. ROSENBAUM. 1971. Comparison of the microtubule proteins of neuroblastoma cells, brain and chlamydomonas flagella. *Proc. Natl. Acad. Sci. U. S. A.* **68**:2273-2277.
- PORZIO, M. A., and A. M. PEARSON. 1977. Improved resolution of myofibrillar proteins with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Biochim. Biophys. Acta.* **490**:27-34.
- RHODIN, J., and T. DALHAMN. 1956. Electron microscopy of the tracheal ciliated mucosa in rat. *Z. Zellforsch. Mikrosk. Anat.* **44**:345-412.
- SHELANSKI, M. L., F. GASKIN, and C. R. CANTOR. 1973. Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. U. S. A.* **70**:765-768.
- SOIFER, D. 1975. The biology of cytoplasmic microtubules. *Ann. N. Y. Acad. Sci.* **253**:5-284.
- SOROKIN, S. P. 1968. Reconstructions of centriole formation and ciliogenesis in mammalian lungs. *J. Cell Sci.* **3**:207-230.
- WICHE, G., and R. D. COLE. 1976. An improved preparation of highly specific tubulin antibodies. *Exp. Cell Res.* **99**:15-22.