Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 2579–2583, April 1982
Cell Biology

Monoclonal antibodies that recognize discrete forms of tubulin

(cell fusion/cytoskeleton/microtubules/heterodimer)

ILLANA GOZES*† AND COLIN J. BARNSTABLE‡§

*Laboratory of Neural and Endocrine Regulation, Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139; and ‡Department of Neurobiology, Harvard Medical School, 25 Shattuck Street, Boston, Massachusetts 02115

Communicated by Torsten N. Wiesel, November 23, 1981

ABSTRACT **Anti-tubulin antibodies secreted by plasmacytoma NSI–spleen cell hybrids were detected by an indirect binding assay. Different antibodies bound to different combinations of the tubulins as resolved by isoelectric focusing. Two monoclonal antibodies (TUB 2.1 and TUB 2.5) labeled only (*i*) the tubulin band on a polyacrylamide electropherogram and (*ii*) β-tubulins as resolved by isoelectric focusing. The fraction that was specifically bound and eluted from antibody affinity columns was enriched in β-tubulins as compared with α-tubulins, suggesting the possibility of some soluble tubulin homodimers and α,β-heterodimers. Double labeling experiments were used to show that all detectable microtubules contained β-tubulin.**

Microtubules are major cytoskeletal elements found in all eukaryotic cells (1). They are composed of a M_r 110,000 protein, tubulin, which is a dimer of two M_r 55,000 subunits (2, 3). Two classes of subunits, α and β, can be identified by a variety of analytical techniques, and it has been suggested on the basis of chemical crosslinking studies that the tubulin dimer is composed of a heterologous pair of these subunits (4).

Different tissues seem to express different tubulins. When examined by isoelectric focusing, liver and spleen had two subunits, one corresponding to α-tubulin and the other to β-tubulin, whereas mature brain had nine subunits, four corresponding to α-tubulin and five to β-tubulin (5). Multiple forms of tubulin also have been described at the protein, mRNA, and DNA level by others (6–13). A series of reagents specific for each form would allow the subcellular localization, metabolism, and possibly the function of each to be studied. Antibodies are ideally suited for such studies. Because the different forms of tubulin cannot be separated readily on a preparative scale and tubulin is poorly immunogenic, such antibodies are unlikely to be produced by conventional immunization. The technique introduced by Milstein and coworkers (14, 15), whereby immune spleen cells are fused with a plasmacytoma cell line to produce antibody-secreting hybrids, has overcome these problems. Monoclonal antibodies already have proved valuable in a variety of biochemical, functional, and genetic studies (16–18).

In this paper we analyze the antigenic complexity of brain tubulin and show that different antibodies bind in distinct patterns to isotubulins separated by isoelectric focusing. Two cloned cell lines produced antibodies against different determinants on β-tubulins. These antibodies have been used to investigate the subcellular localization of β-tubulins and to reexamine the molecular nature of the M_r 110,000 tubulin dimer.

MATERIALS AND METHODS

Preparation of Immunogen and Immunization. Tubulin was partially purified from adult Sprague–Dawley rat brains by one cycle of *in vitro* assembly (19). Female BALB/c mice were injected subcutaneously at 3-wk intervals with 100–500 μg of tubulin emulsified in Freund's adjuvant; 8 out of 10 mice gave a measurable anti-tubulin response. The mouse giving the greatest response was boosted with an intravenous injection of 50 μg of tubulin in saline.

Fusion and Cloning. Four days after intravenous injection, spleen cells were isolated and fused with plasmacytoma cell line P3 NSI/1-4Ag-1 as described by Galfre *et al.* (15). After fusion, the cell suspension was divided into 144 samples in RPMI 1640 medium containing 10% fetal calf serum and hypoxanthine/aminopterin/thymidine (20). Supernatants were tested for antibody activity after 14 days. Positive cultures were adapted onto medium without hypoxanthine/aminopterin/thymidine, grown to larger volumes, frozen, and stored in liquid N_2.

Clones were isolated from cultures that were plated at less than one cell per well and subsequently cloned in 0.3% agar (21, 22).

Antibody Binding Assays. Formaldehyde-fixed sheep erythrocytes were activated by tannic acid (23) and then coated with tubulin purified by DEAE-cellulose chromatography (24, 25) at a coupling ratio of 200 μg of tubulin per ml of packed cells. Assay of sera or culture supernatants was carried out as described (18, 26) except that microwell plates (Dynatech) and a final volume of 50 μl were used. Cells finally were harvested onto glass-fiber filter mats with a semiautomated cell harvester (Dynatech CH103), and bound radioactivity was assayed in a gamma counter.

Indirect Immunofluorescence. Dissociated neurons from the Sprague–Dawley rat superior cervical ganglion were grown in the absence of nonneuronal cells (27). Nonneuronal cells from a similar origin were grown in the absence of both nerve growth factor and antimitotic agents. Secondary fibroblast cultures (NRF-1) were obtained from trypsinized newborn-rat lungs (CD; Charles River Breeding Laboratories) (28).

Cells were grown on coverslips, washed in phosphate-buffered saline (hereafter referred to as saline) and fixed for 15 min at room temperature with 1% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.5). After rinsing in saline alone and with 1% bovine serum albumin, cells were treated with antibody diluted in saline containing 0.05% Triton X-100. The coverslips were incubated for 1 hr at room temperature, washed three times in saline/Triton X-100, and then treated with rhodamine-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) in saline/Triton X-100/1% bovine serum albumin. After 1 hr at room temperature, they were washed three times in saline/Triton X-100 and mounted on slides. For double-labeling experiments, the coverslips were incubated first with the monoclonal antibody and then with a 1:10 dilution of rabbit anti-tubulin antiserum (25). After washing, the coverslips were incubated with fluorescein-conjugated goat anti-rab-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

† Present address: A. V. Davis Center for Behavioral Neurobiology, The Salk Institute, P.O. Box 85800, San Diego, CA 92138.
§ To whom reprint requests should be addressed.

bit IgG and rhodamine-conjugated goat anti-mouse IgG. Control experiments established that neither labeled antiserum showed heterologous crossreactivity.

Gel Electrophoresis. *Sodium dodecyl sulfate/polyacrylamide gel electrophoresis.* The buffer system of Laemmli (29) was used. Samples (10 μg) were subjected to electrophoresis at 3 V/cm at 20°C for 14–16 hr on 0.75-mm thick polyacrylamide gel slabs containing 0.1% $NaDodSO_4$.

Isoelectric focusing. The technique was carried out as described by O'Farrell (30); 10 μg of DEAE-cellulose-purified tubulin was applied per gel (14–16 × 0.25 cm).

Binding of Antibodies to Tubulin Separated on Polyacrylamide Gels. *Indirect labeling.* The method described by Burridge (31) was used with slight modifications. Fixed and neutralized gels were incubated with culture supernatant and, after washing, the bound antibody was detected by either ^{125}I-labeled $F(ab')_2$ rabbit anti-mouse IgG fragment (0.05–0.1 μg per gel) or by ^{125}I-labeled protein A diluted in 5% normal rabbit serum in buffer A: 0.9% NaCl/50 mM Tris·HCl, pH 7.5/0.1% NaN_3 (28). Specific activities were 20–40 μCi/μg (1 Ci = 3.7 × 10^{10} becquerels).

Direct labeling. Cell lines were labeled with [^{35}S]methionine (6–8 mCi/ml; 900–1000 Ci/mmol, Amersham) for 18 hr. Tubulin-containing gels were each incubated with 0.3 ml of radioactive culture media that had been diluted with buffer A and 5% normal rabbit serum to contain radioactive trichloroacetic acid-insoluble material at 0.6 × 10^6 cpm/0.3 ml.

Autoradiography. Gels were stained with Coomassie brilliant blue, dried *in vacuo*, and exposed to Kodak XR x-ray film and Dupont Cronex Lightning-Plus intensifying screens (32).

Affinity Chromatography. Antibodies were partially purified from ascites fluid by ammonium sulfate precipitation and coupled to cyanogen bromide-activated Sepharose 4B (16) at a ratio of 5 mg of protein to 1 ml of settled gel. Columns were prewashed with 50 mM diethylamine·HCl (pH 10.5) and then equilibrated with saline. The columns were loaded with a supernatant (16,000 × g) of CD rat cerebral cortex homogenized in sucrose or phosphate buffer, allowed to equilibrate, and then washed with saline until no further protein was eluted. The columns were eluted with 50 mM diethylamine·HCl (pH 10.5). Fractions were neutralized, concentrated by ultrafiltration, and dialyzed against water prior to analysis.

RESULTS

Production of Anti-Tubulin Antibodies. Fourteen days after the fusion, almost all wells contained growing cultures, and supernatants from these were assayed for anti-tubulin antibodies. Twenty out of 144 cultures gave a positive response (defined as 2 times the background), and 5 of these were strongly positive (5 times the background). Selected culture supernatants were tested for their ability to bind to brain tubulin that had been subjected to isoelectric focusing. Some of the supernatants (3 out of 15) showed no detectable binding, and others bound to essentially all of the forms of tubulin resolved in this procedure (3 out of 15). However, some bound to a restricted number of forms of tubulin (Fig. 1). For example, the supernatant in Fig. 1*C*, lane c, bound isotubulin 3, whereas that in lane g bound isotubulins 4, 5, 6, and 7, and that in lane i bound isotubulins 6, 7, and 8. Because the supernatants used were not from cloned cells, it is not clear whether binding to several bands on the gel was due to the presence of heterogeneous cells or, more likely, to antigenic determinants shared between the various forms of the molecule.

Two cultures (TUB 2.1 and TUB 2.5) that appeared to give strong binding in the early screening assays were cloned by limiting dilution, followed by cloning in soft agar. Both were IgG antibodies—TUB 2.1 was of the γ_1 subclass and TUB 2.5 was of the γ_3 subclass.

Monoclonal Antibodies That Recognize Discrete Forms of Tubulin. The two cloned antibodies labeled only the tubulin M_r 55,000 band in a rat brain protein extract run on a $NaDodSO_4$/polyacrylamide electropherogram (Fig. 2). In the absence of mouse anti-tubulin antibodies, no binding of ^{125}I-labeled rabbit anti-mouse or ^{125}I-labeled protein A was detected (Fig. 2*A*).

The two cloned antibodies labeled bands 5–9 on the isoelectric focusing gels that represent the β-tubulins (5), although different bands were labeled to different intensities by the two antibodies (Fig. 3). These apparent differences were reproducible in four independent experiments and were not related to any differences in the amount of protein present in each band. TUB 2.5 also bound to some extent to bands 3 and 4. In a binding assay, TUB 2.1 did not block the binding of TUB 2.5, and vice versa. Thus, the antibodies seem to bind different determinants on the immobilized tubulin.

Tubulin Subunit Associations. The supernatant (16,000 × g) from homogenized rat cerebral cortex was applied to columns of monoclonal anti-tubulin antibodies coupled to Sepharose 4B. The material that was bound to and eluted from columns of TUB 2.1 or TUB 2.5 was also analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 4, lanes b and c). Enrichment for M_r 55,000 polypeptides migrating identically with purified α-

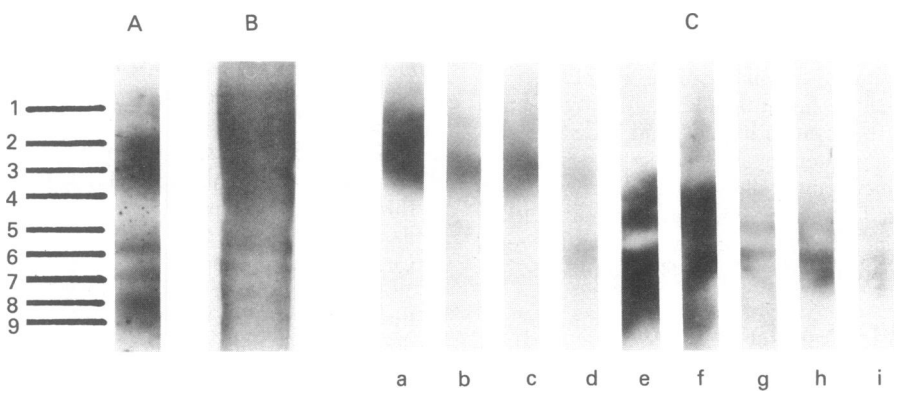

FIG. 1. Binding of anti-tubulin culture supernatants to tubulin separated on isoelectric focusing gels. Rat brain tubulin was purified by DEAE-cellulose chromatography and separated on isoelectric focusing gels. Gels were incubated with culture supernatants for indirect labeling. (*A*) Coomassie brilliant-blue stain. (*B*) Autoradiogram of mouse anti-tubulin serum diluted 1:30 (vol/vol) in buffer A. (*C*) Lanes: a–j: autoradiograms of supernatants from different cultures. Bands 1–4 correspond to α-tubulin and bands 5–9, to β-tubulin (5). The separation between isotubulin bands 2 and 4 is not as distinct as between bands 6 and 8 as noted (5).

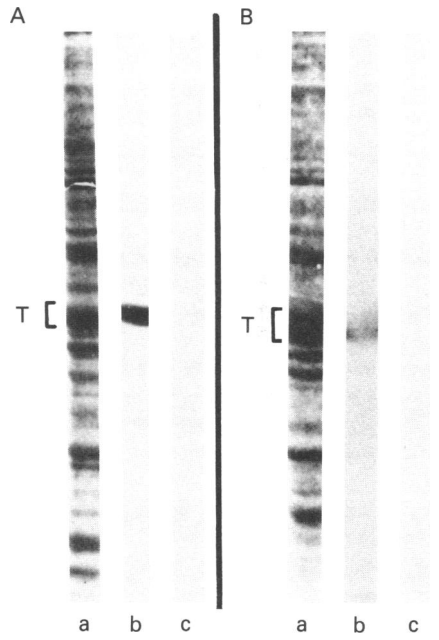

FIG. 2. Binding of monoclonal anti-tubulin antibodies to tubulin (T) separated on $NaDodSO_4$/polyacrylamide gels. (*A*) Brain supernatant (105,000 × *g*) was electrophoresed on 10% polyacrylamide/0.1% $NaDodSO_4$ gels, which were then processed for indirect labeling. Lanes: a, Coomassie brilliant blue stain; b, autoradiogram of TUB 2.1 antibody and ^{125}I-labeled $F(ab')_2$ rabbit anti-mouse IgG; c, autoradiogram of control with no first antibody and ^{125}I-labeled $F(ab')_2$ rabbit anti-mouse IgG. (*B*) Brain supernatant (30,000 × *g*) prepared in the presence of phenylmethylsulfonyl fluoride (10 μg/ml), pepstatin A (10 μg/ml), and kallikrein inactivator (20 μg/ml) was electrophoresed on 8.65% polyacrylamide/0.1% $NaDodSO_4$ gels. The gels were then processed for indirect labeling by using ^{125}I-labeled protein A (1 μCi per gel; New England Nuclear) diluted 5% with bovine serum albumin in buffer A. Lanes: a, Coomassie brilliant blue stain; b, autoradiogram of TUB 2.5 antibody and ^{125}I-labeled protein A; c, autoradiogram of control using no first antibody and ^{125}I-labeled protein A. It is interesting to note that TUB 2.5 labels mainly the lower part of the tubulin band, probably corresponding to the β-tubulin subunit that sometimes separates from the α subunit on $NaDodSO_4$/polyacrylamide gels (see Figs. 3 and 4).

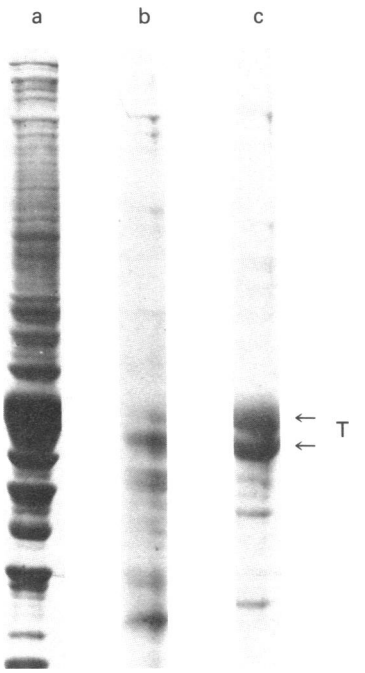

FIG. 4. Isolation of tubulin by affinity chromatography. Brain supernatant (105,000 × *g*) was passed over columns of monoclonal anti-tubulin antibody coupled to Sepharose 4B, and the columns were washed and eluted. The specifically bound and eluted material was analyzed on 8.5% polyacrylamide gels at a pH of 9.3 to resolve α- and β-tubulins. The gels were stained, and the OD_{560} in the tubulin region was determined. From the scans, the relative amounts of material in the α- and β-tubulin peaks were determined. The arrows indicate the tubulin bands. Lanes: a, brain supernatant loaded onto the column; b, material bound to TUB 2.1; c, material bound to TUB 2.5.

and β-tubulins was observed and, in addition, the ratio of α-tubulin to β-tubulins differed from that of the starting material. Ratios (β/α) of 3.7:1 for TUB 2.1 and 1.22:1 for TUB 2.5 were obtained from densitometric scans of the gel shown in Fig. 4, lanes b and c, as compared with 0.8:1 for the gel in Fig. 4, lane a. The bands of molecular weight lower than that of tubulin are unlikely to be due to proteolytic breakdown products because they also were found when buffers saturated with phenylmethylsulfonyl fluoride and containing 1 mM EDTA were used.

The additional bands on the gels shown in Fig. 4, lanes b and c, were probably due to nonspecific binding. Treatments that have been shown to decrease the amount of nonspecific binding to antibody affinity columns (33) would have enhanced the de-

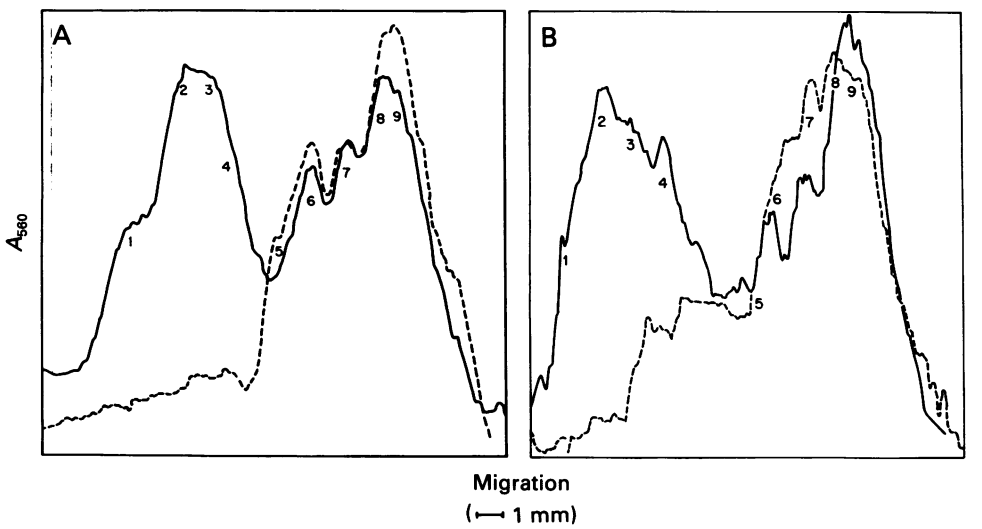

FIG. 3. Binding of monoclonal anti-tubulin antibodies to tubulin separated on isoelectric focusing gels. Rat brain tubulin was purified by DEAE-cellulose chromatography and separated on isoelectric focusing gels. [^{35}S]Methionine-labeled culture media were incubated with the fixed and neutralized gels for 24 hr. The gels were stained with Coomassie brilliant blue and autoradiographed. In each case, both the scans of the protein stain (——) and the autoradiogram (----) are shown. The numbers represent the individual tubulins as in Fig. 1. (A) TUB 2.1. (B) TUB 2.5.

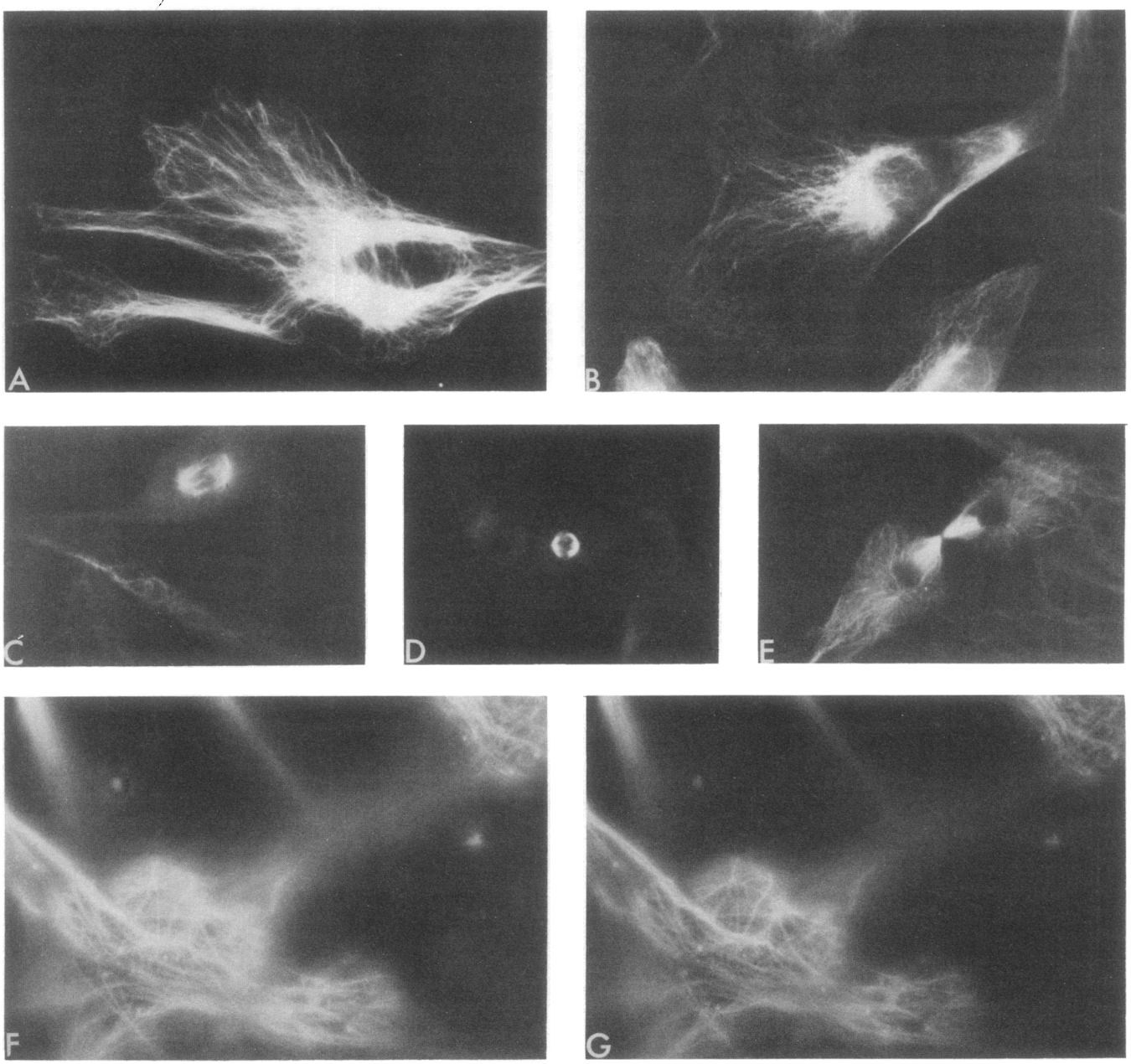

FIG. 5. Subcellular distribution of β-tubulin as studied by indirect immunofluorescence. Labeling of interphase (*A* and *B*), mitotic (*C* and *D*), and late telophase (*E*) cells by antibodies TUB 2.5 (*A*, *C*, and *E*) or TUB 2.1 (*B* and *D*) and rhodamine-conjugated goat anti-mouse IgG. Superior cervical ganglion cells after 3 wk in culture were labeled with either rabbit anti-tubulin antibodies and fluorescein-conjugated goat anti-rabbit IgG or TUB 2.5 and rhodamine-conjugated goat anti-mouse IgG. The same field is shown with fluorescein (*F*) or rhodamine (*G*) optics.

gree of purification but also might have influenced the tubulin subunit associations.

Subcellular Distribution of β-Tubulins. When fibroblastic cells were stained with either TUB 2.1 or TUB 2.5, they showed a pattern typical of microtubules (Fig. 5 *A* and *B*)—that is, a perinuclear network and a system of fibers throughout the cell but not extending into tips of the cell filopodia. Both antibodies labeled mitotic spindles and the area round the cleavage furrow of these cells (Fig. 5 *C–E*). When double-labeling experiments were carried out with the monoclonal antibodies and a rabbit serum directed against both α- and β-tubulin (25), no significant differences were found, indicating that no major microtubular structures exist in fibroblastic cells that do not contain β-tubulin—i.e., stained with whole serum but not cloned antibody.

Cultured sympathetic neurons showed microtubule networks in both the cell bodies and axons, as shown for TUB 2.5 (Fig. 5*G*). Again double-labeling experiments (Fig. 5 *F* and *G*) failed to reveal any major structures that did not contain β-tubulin.

DISCUSSION

In this paper, we show that monospecific antibody reagents can be used to identify various forms of tubulin as defined by biochemical criteria and then to relate those forms to particular subcellular structures. Various antigenic determinants are shared between different combinations of tubulins that can be resolved by isoelectric focusing (Fig. 1). Thus, the microheterogeneity found by isoelectric focusing of purified tubulin has a structural basis that can be detected immunologically. The two monoclonal antibodies described in more detail in this paper

labeled only the β-tubulins (Fig. 3), although the ratios of labeling of the various resolved bands differed somewhat between the two antibodies.

The monoclonal anti-β-tubulin antibodies have been used to examine the nature of the tubulin dimer. The α/β ratio of material bound and eluted from columns of anti-β-tubulin antibody was significantly enriched in β-tubulin over the starting material. Had all the tubulin in the starting sample been in the form of α,β-tubulin dimers, then the α/β ratio in the eluted material should have been the same as in the starting material. It is possible that, upon binding of β-tubulin to antibody, the α–β association is disrupted, causing a preponderance of β-tubulin monomers to be retained by the column. This, we feel, is unlikely because the antibodies do not inhibit tubulin–tubulin interactions during *in vitro* microtubule assembly (34). Spontaneous dissociation of α,β-heterodimers (35), leaving the β-subunit bound to the column, is also a possibility, although altering the column wash volume by a factor of 4 did not significantly alter the ratio of eluted α and β subunits. Thus, taken with the evidence for heterodimers given by Luduena *et al.* (4), it is possible that the tubulin monomers are in a state of dynamic equilibrium and that both homodimers and heterodimers are present in significant quantities.

This, together with the increasing evidence for multiple tubulin gene products, suggests that many different forms of tubulin may be available for polymerization into microtubular structures. Further, if the multiple tubulin gene products are functionally different, rather than merely representing neutral divergence of the duplicated genes, then control of microtubule-mediated cell metabolism might be exerted by selective gene expression. Although we found no evidence for cellular structures lacking β-tubulin, the presence or absence of particular β-tubulins could not be assessed. The resolving power of monoclonal antibodies directed against individual tubulin gene products will allow a more precise investigation of the subcellular localization and functional interrelationships of tubulins in complex cellular arrays such as the brain.

We thank M. Moskowitz and T. Wiesel for their encouragement and support, K. Sweadner and P. Patterson for their help with the neural cultures, B. Cronin and L. Mangini for technical assistance, and K. Cross for photography. This work was supported by a Chaim Weizmann Fellowship to I.G., Damon Runyon–Walter Winchell Cancer Fund Fellowship DRG-242 and Grants EY01995 and NS 17309 to C.J.B., and grants from the National Institutes of Health to M.M. and T.W. and from the Sloan Foundation to the Harvard Neurobiology Department.

1. Dustin, P. (1978) *Microtubules* (Springer, Berlin).
2. Weisenberg, R. C., Borisy, G. G. & Taylor, E. W. (1968) *Biochemistry* **7**, 4466–4479.
3. Bryan, J. & Wilson, L. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 1762–1766.
4. Luduena, R. F., Shooter, E. M. & Wilson, L. (1977) *J. Biol. Chem.* **252**, 7006–7014.
5. Gozes, I. & Littauer, U. Z. (1978) *Nature (London)* **276**, 411–413.
6. Marotta, C. A., Harris, J. L. & Gilbert, J. M. (1978) *J. Neurochem.* **30**, 1431–1440.
7. Nelles, L. P. & Bamburg, J. R. (1979) *J. Neurochem.* **32**, 477–489.
8. Dahl, J. L. & Weibel, V. J. (1979) *Biochem. Biophys. Res. Commun.* **86**, 822–828.
9. Marotta, C. A., Strocchi, P. & Gilbert, J. M. (1979) *J. Neurochem.* **33**, 231–246.
10. Gozes, I., de Baetselier, A. & Littauer, U. Z. (1980) *Eur. J. Biochem.* **103**, 13–20.
11. Cleveland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J. & Kirschner, M. W. (1980) *Cell* **20**, 95–105.
12. Alexandraki, D. & Ruderman, J. V. (1979) *J. Cell Biol.* **83**, 342 (abstr.).
13. Ginzburg, I., de Baetselier, A., Walker, M. D., Behar, L., Lehrach, H., Frischaus, A. N. & Littauer, U. Z. (1980) *Nucleic Acids Res.* **8**, 3553–3564.
14. Köhler, G. & Milstein, C. (1975) *Nature (London)* **256**, 495–497.
15. Galfre, C., Howe, S. C., Milstein, C., Butcher, G. W. & Howard, J. C. (1977) *Nature (London)* **266**, 550–552.
16. Parham, P., Barnstable, C. J. & Bodmer, W. F. (1979) *J. Immunol.* **123**, 342–349.
17. White, R. A. H., Mason, D. W., Williams, A. F., Galfre, G. & Milstein, C. (1978) *J. Exp. Med.* **148**, 666–673.
18. Barnstable, C. J., Bodmer, W. F., Brown, C., Galfre, G., Milstein, C., Williams, A. F. & Ziegler, A. (1978) *Cell* **14**, 9–20.
19. Fellous, A., Francon, J., Lennon, A.-M. & Nunez, J. (1977) *Eur. J. Biochem.* **78**, 167–174.
20. Littlefield, J. W. (1964) *Science* **145**, 709–710.
21. Cotton, R. G. H., Secher, D. S. & Milstein, C. (1973) *Eur. J. Immunol.* **3**, 135–140.
22. Brodsky, F. M., Parham, P., Barnstable, C. J., Crumpton, M. J. & Bodmer, W. F. (1979) *Immunol. Rev.* **47**, 3–61.
23. Herbert, W. J. (1967) in *Handbook of Experimental Immunology*, ed. Weir, D. M. (Blackwell, Oxford), 720–744.
24. Eipper, B. A. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2283–2287.
25. Gozes, I., Littauer, U. Z., Geiger, B. & Fuchs, S. (1977) *FEBS Lett.* **73**, 109–114.
26. Williams, A. F. (1977) *Contemp. Top. Mol. Immunol.* **6**, 93–116.
27. Hawrot, E. & Patterson, P. H. (1979) *Methods Enzymol.* **53**, 574–584.
28. Barnstable, C. J. (1980) *Nature (London)* **286**, 231–235.
29. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
30. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021.
31. Burridge, K. (1978) *Methods Enzymol.* **50**, 54–64.
32. Laskey, R. A. & Mills, A. D. (1977) *FEBS Lett.* **82**, 314–316.
33. Snary, D., Barnstable, C. J., Bodmer, W. F., Goodfellow, P. N. & Crumpton, M. J. (1977) *Scand. J. Immunol.* **6**, 439–452.
34. Barnstable, C. J. & Gozes, I. (1980) *J. Cell Biol.* **87**, 251 (abstr.).
35. Dietrich, H. W. & Williams, R. C. (1978) *Biochemistry* **17**, 3900–3907.