A Radiolabeled Monoclonal Antibody Binding Assay for Cytoskeletal Tubulin in Cultured Cells

Rebecca L. Ball,* Darrell H. Carney,‡ Thomas Albrecht,* David J. Asai,§ and William C. Thompson‡

*Department of Microbiology and ‡Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77550; and §Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

Abstract. To detect changes in the extent of tubulin polymerization in cultured cells, we have developed a radioactive antibody binding assay that can be used to quantitate total cytoskeletal tubulin or specific antigenic subsets of polymerized tubulin. Fibroblastic cells, grown to confluence in multiwell plates, were permeabilized and extracted with 0.5% Triton X-100 in a microtubule-stabilizing buffer. These extracted cytoskeletons were then fixed and incubated with translationally radiolabeled monoclonal antitubulin antibody (Ab 1-1.1), an IgM antibody specific for the beta subunit of tubulin. Specific binding of Ab 1-1.1 to the cytoskeletons was saturable and of a single apparent affinity. All specific binding was blocked by preincubation of the radiolabeled antibody with excess purified brain tubulin. Specific Ab 1-1.1 binding appeared to represent binding to cytoskeletal tubulin inasmuch as: (*a*) pretreatment of cells with colchicine decreased Ab 1-1.1 binding in a dose-dependent manner which correlated with the amount of polymerized tubulin visualized in parallel cultures by indirect immunofluorescence, (*b*) taxol pretreatment alone caused an increase in Ab 1-1.1 binding and prevented in a dose-dependent manner the colchicine-induced decrease in antibody binding, (*c*) in cells pretreated with colcemid and returned to fresh medium, Ab 1-1.1 binding decreased and recovered in parallel with the depolymerization and regrowth of microtubules in these cells, and (*d*) comparison of maximal antibody binding per cell between primary mouse embryo, 3T3, and human foreskin fibroblasts correlated with immunofluorescence visualization of microtubules in these cells. Thus, this assay can be used to measure relative changes in the level of polymerized cytoskeletal tubulin. Moreover, by Scatchard-type analysis of the binding data it is possible to estimate the total number of antibody binding sites per cell. Therefore, depending on the stoichiometry of antibody binding, this type of assay may be used for quantitating total cytoskeletal tubulin, specific antigenic subsets of cytoskeletal tubulin, or other cytoskeletal proteins.

MICROTUBULES are tubular polymers whose protomeric unit is a heterodimer consisting of α- and β-tubulin subunits (for a recent review see reference 20). The cytoplasm of eukaryotic cells contains an organized array of microtubules as well as a soluble pool of unpolymerized tubulin protomers. These cytoplasmic microtubules represent a dynamic element of the cytoskeleton since microtubules can be rapidly assembled or disassembled in response to various stimuli with little or no net change in the total tubulin content of the cell (24). Inoué and Sato (17) demonstrated that the polymer and protomer forms of intracellular tubulin are in a "dynamic equilibrium" which can be perturbed by changes in pressure. This equilibrium can also be perturbed by drugs such as colchicine and vinblastine which induce microtubule disassembly in vivo (41), or by taxol, which stabilizes microtubules and promotes the assembly of supernumerary microtubules within the cytoplasm (21, 37).

Cytoplasmic microtubules have been implicated in such diverse processes as cellular motility, intracellular transport, secretion, organization of the cytoplasm, organization of proteins in the membrane, growth factor signalling, and may undergo changes related to transformation (6, 10, 11, 12–14, 23). It has long been of interest to determine if changes in the extent of polymerization of tubulin are associated with such processes. Estimates of cytoskeletal tubulin can be made either directly, by binding reagents specifically to microtubules, or indirectly, by subtracting the amount of unpolymerized tubulin from the total tubulin determined after depolymerization. It has been suggested that cytoskeletal tubulin can be quantitated directly by binding radiolabeled taxol to microtubules (27, 28), but an effective labeled reagent is difficult to prepare and may bind differentially to certain classes of microtubules (29). In addition, immunofluorescence techniques with antitubulin antibodies have been used to directly visualize cellular microtubules to document

gross changes in microtubule content (6, 41). However, immunofluorescence staining of cytoskeletal structures has proved difficult to quantitate, particularly for large populations of cells.

Among the indirect methods of determining cytoskeletal tubulin, several protocols have been developed that use the binding of labeled colchicine to free tubulin promoters to estimate the unpolymerized tubulin pool and the total tubulin in a tissue. Cytoskeletal tubulin is then estimated as the difference between these values (3, 4, 22, 25, 26, 30–33, 40). Traditional radioimmunoassays for unpolymerized tubulin have also been developed (15, 16, 18, 19, 39) that could theoretically be used to estimate the cytoskeletal tubulin content of cells by a subtractive method.

We describe here a direct radioactive antibody binding assay specific for cytoskeletal tubulin in cultured cells. Distinction between polymerized and unpolymerized tubulin is made by detergent-extraction of the unpolymerized tubulin pool and other soluble cytoplasmic components before antibody binding to the insoluble cytoskeletons. To ensure that the labeled binding reagent has a single affinity for tubulin, we have used a monoclonal antitubulin antibody (Ab 1-1.1) which has been previously shown by indirect immunofluorescence to bind to all microtubules of the cytoplasmic microtubule complex (37). By incorporating other, more discriminatory, antibody reagents, the procedure described here should also prove invaluable for examining the partitioning of antigenically unique species of tubulin within cellular pools (37).

Materials and Methods

Materials

Except as otherwise noted, reagent grade chemicals from Sigma Chemical Co. (St. Louis, MO) were used in these experiments. Taxol was provided by Dr. Matthew Suffness, Natural Products Branch, National Cancer Institute, and was diluted in ethanol. Colchicine and colcemid (demecolcine) were diluted in PBS without divalent cations. Polyacrylamide gel components were obtained from Bio-Rad Laboratories (Richmond, CA), except for the 95% pure grade of sodium lauryl sulfate (Sigma Chemical Co.). [^{35}S]Methionine was purchased from Amersham Corp. (Arlington Heights, IL) and ^{3}H-amino acid mixture from ICN Radiochemicals (Div. ICN Biomedicals Inc., Irvine, CA). Horse serum was obtained from KC Biological Inc. (Lenexa, KS), calf serum from Irvine Scientific (Santa Ana, CA), and culture medium and supplements from Gibco (Grand Island, NY).

Antibodies

The monoclonal antitubulin Ab 1-1.1 used in this study has previously been shown by double-label immunofluorescence to bind to all of the cytoplasmic microtubules in mouse fibroblastic cells (37). As previously described, the hybridoma clone secreting Ab 1-1.1 was derived from the fusion of a nonsecreting murine myeloma cell and an immune splenocyte from a BALB/c mouse immunized with sea urchin axonemal proteins (1, 2). Using an ELISA antibody typing kit (Boehringer Mannheim Biochemicals, Indianapolis, IN), we determined Ab 1-1.1 to be an immunoglobulin of the IgM (kappa) class. Ascites serum containing high concentrations of unlabeled Ab 1-1.1 was obtained from pristane-primed BALB/c female mice bearing ascites tumors of the 1-1.1 hybridoma clone. For use in some experiments, as noted, an IgM fraction was isolated from the ascites fluid (5).

Monoclonal antibody was translationally radiolabeled and secreted into the culture medium by cells of the 1-1.1 hybridoma clone during growth for 24–30 h in RPMI 1640 medium supplemented with 15% horse serum and containing 0.05 mCi/ml of [^{35}S]methionine or ^{3}H-amino acid mixture. Cells were pelleted by centrifugation for 10 min at 1,000 *g*, and the supernatant medium was removed, dialyzed against several changes of 100 vol of PBS (Dulbecco's phosphate-buffered saline without divalent cations), filter sterilized, and used directly as a source of labeled Ab 1-1.1. Alternatively, an IgM fraction was purified from the dialyzed culture supernatant by Sephacryl S-200 chromatography as described by Bouvet et al. (5). This purified reagent was dialyzed overnight against PBS, filter sterilized, and stored at 4°C for use as a stock of labeled Ab 1-1.1 for binding to microtubules in extracted cytoskeletons.

Immunoblotting

Phosphocellulose-purified, thrice-cycled, mouse brain tubulin and total mouse embryo (ME)[1] cellular proteins were taken up in sample buffer and subjected to electrophoresis on 8% polyacrylamide gels according to the formulations of Sheir-Neiss et al. (36). The resolved protein pattern was transferred by Western blotting (7, 38) to nitrocellulose paper (grade BA85, Schleicher & Schuell, Keene, NH). It was necessary to remove the SDS by soaking the nitrocellulose blot briefly in 1% Triton X-100 in PBS to allow efficient binding of Ab 1-1.1 to the denatured tubulin (suggested by Dr. Eugenia Wang, Rockefeller University). The nitrocellulose was blocked by incubation for 1 h in a solution of 10% dried milk solids (Carnation) in Tris-buffered saline (TBS): 150 mM NaCl, 10 mM Tris, pH 7.5. The nitrocellulose blot was then incubated for 2 h at room temperature with hybridoma culture supernatant containing Ab 1-1.1, rinsed, and incubated with peroxidase-conjugated goat anti-mouse antibodies (anti-IgG, -IgA, -IgM) obtained from Cappel Laboratories (Cochranville, PA). After a final rinse with Tris-buffered saline, the pattern of bound antibody was developed by incubation with 0.07% 4-chloro-1-naphthol (Bio-Rad horseradish peroxidase color development reagent) and 0.02% H_2O_2 in a solution of 20% methanol and 80% Tris-buffered saline.

Cell Culture

Clone 1-1.1 murine hybridoma cells were cultured in plastic tissue culture flasks (75 cm^2) in RPMI 1640 medium supplemented with 15% horse serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere containing 7.5% CO_2. Primary cultures of ME fibroblastic cells, obtained by trypsinization of the body wall of 9–11-d embryos as described (8), were cultured in DV-F12 medium (45% Dulbecco-Voght modified Eagle's medium, 45% Ham's F12 medium, 10% calf serum, and antibiotics as above) in a humidified atmosphere containing 5% CO_2. Human foreskin (HF) cells, prepared as described (8), and mouse 3T3 cells were also maintained in DV-F12 medium. Secondary cultures of these fibroblastic cells were plated either in 24- or 48-well tissue culture plates at an initial density of 5×10^4 cells/cm^2. In most experiments, wells were precoated with 2% gelatin. Because we are interested in using this assay for growth control studies in the future, confluent cultures of these fibroblastic cells were incubated in serum-free DV-F12 medium for 48 h to bring the cells to a quiescent G_0 state before the binding analysis.

Binding of Radioactive Antibody to Cytoskeletal Tubulin

Confluent cultures of nonproliferating fibroblasts in multiwell culture plates were rinsed with warm microtubule-stabilizing buffer (MSB) containing 100 mM Pipes (pH 6.9), 1 mM EGTA, and 4% polyethylene glycol (PEG-3350). The cell membranes were then permeabilized by incubation for 10 min at room temperature in 0.5% Triton X-100 in MSB supplemented with the proteolytic inhibitor aprotinin (0.1 trypsin inhibitor units/ml). The resulting cytoskeletons were rinsed three times in warm MSB to remove free tubulin, fixed for 10 min in a solution of 4% formaldehyde (freshly prepared from paraformaldehyde) in warm MSB, and rehydrated in PBS before antibody binding. This fixation procedure was found to be superior to methanol fixation.

Fixed cytoskeletons were incubated with radiolabeled Ab 1-1.1 (diluted in PBS containing 0.05% Tween 20) at 4°C overnight with continuous gentle agitation. After extensive washing of the culture plate in PBS to remove unbound antibody, the bound radioactive antibody was released into 1.0 N NaOH at room temperature for 24 h. Samples were neutralized with HCl and were counted in 10 ml of Ready Solve MP-B (Beckman, Houston, TX). For determination of the nonspecific binding, 10 or 20 μl of homologous ascites serum containing a large excess of unlabeled Ab 1-1.1 was added to wells of 48- or 24-well plates, respectively, immediately before addition of the labeled antibody.

1. *Abbreviations used in this paper*: HF, human foreskin; ME, mouse embryo; MSB, microtubule-stabilizing buffer.

Specific Activity of Radiolabeled Ab 1-1.1

An IgM fraction containing radiolabeled Ab 1-1.1 was isolated from hybridoma 1-1.1 culture supernatant by the method of Bouvet et al. (5). In this procedure, the supernatant proteins are resolved by gel permeation chromatography on Sephacryl S-200 preequilibrated with a low ionic strength buffer and eluted with a high ionic strength buffer. IgM molecules, being euglobulins, are not soluble in the low ionic strength buffer and, instead of eluting at the excluded volume, elute at the included volume with the high ionic strength buffer. The radioactive protein in this fraction co-migrates with the IgM heavy and light chains, but the fraction still contains some unlabeled serum proteins. Therefore, we have determined the protein content of only the active labeled Ab 1-1.1 molecules using data from saturation curves and competitive binding experiments where the concentration of highly purified unlabeled Ab 1-1.1 is known. By determining the amount of unlabeled antibody necessary to decrease the specific activity of the bound antibody by two-thirds, we calculated the concentration of active Ab 1-1.1 protein in the labeled supernatant fraction. The supernatant used for quantitation studies contained 101 μg of active antibody per milliliter with a specific activity of 5.26×10^3 cpm/μg.

Immunofluorescence

For correlation of the binding experiments with indirect immunofluorescence staining, cells were grown on glass coverslips (8.3×10^3 cells/cm^2) and brought to quiescence as described above. Cell membranes were permeabilized and the resulting cytoskeletons fixed as in antibody binding assays. The coverslips were then rinsed in Ca^{++}-, Mg^{++}-free PBS and the cells incubated with one drop of undilute unlabeled hybridoma 1-1.1 supernatant for 1 h at 37°C. After incubation, the coverslips were rinsed extensively in PBS and incubated as above with a fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). The coverslips were rinsed again in PBS and mounted on glass slides in 1 drop of 10% glycerol in borate saline. Cells were examined with a Leitz Orthoplan microscope with epifluorescent illumination and were photographed using Kodak Tri-X pan film pushed to ASA 1600 with ethol blue.

Results

Characterization of Ab 1-1.1

For the development of this antibody binding assay for cytoskeletal tubulin, we have used an IgM monoclonal antitubulin antibody designated Ab 1-1.1. The hybridoma clone secreting Ab 1-1.1 was derived from the fusion of an M5 murine myeloma cell and a splenocyte from a mouse previously immunized with an extract of sea urchin (*Strongylocentrotus purpuratus*) sperm tail axonemal proteins as described (1, 2). This antibody has been shown by immunofluorescence studies to bind to all microtubules in the cytoplasmic microtubule complex of mouse fibroblasts (37). To determine if this monoclonal antibody might bind to proteins other than tubulin, purified tubulin and total ME cell proteins were subjected to electrophoresis, blotted to nitrocellulose, and immunostained. As shown in Fig. 1, Ab 1-1.1 binds specifically to the beta subunit of purified brain tubulin (lane *A'*). Likewise, when total mouse fibroblast proteins were immunostained with Ab 1-1.1, only a single band corresponding to the beta-tubulin subunit was labeled (lane *B'*).

Binding of Radiolabeled Ab 1-1.1 to Cytoskeletons

To obtain a translationally radiolabeled antibody probe for microtubules, we incubated log phase hybridoma 1-1.1 cells in complete RPMI 1640 medium containing labeled amino acids. The supernatant culture fluid was then dialyzed extensively against PBS, filter sterilized, and used directly as a source of radioactive antibody for binding experiments, or an IgM fraction isolated from hybridoma 1-1.1 supernatant (5) was used.

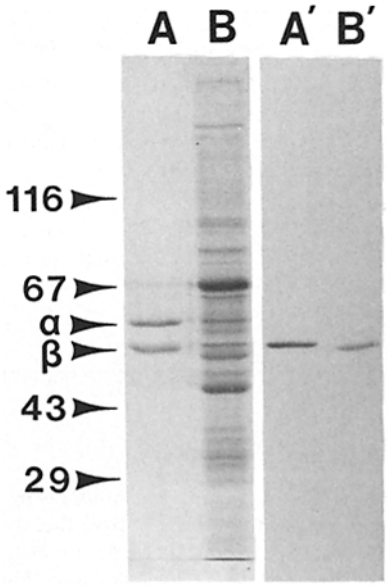

Figure 1. Monoclonal Ab 1-1.1 binds to the beta subunit of tubulin. ME fibroblastic cells were rinsed with PBS and dissolved on the plate in SDS electrophoresis sample buffer, sonicated to fragment the DNA, and heated at 100°C for 2 min. Mouse brain microtubule protein purified by three cycles of polymerization was pelleted through 50% sucrose and separated from MAPs by phosphocellulose chromatography. Purified brain tubulin (25 μg, lane *A*) and total ME cell proteins (lane *B*) were subjected to electrophoresis on 8% polyacrylamide (SDS) slab gels that were either stained with Coomassie Blue or Western blotted to nitrocellulose. The nitrocellulose sheet was soaked for 5 min in 1% Triton X-100 in PBS to remove the SDS, blocked, and immunostained with monoclonal Ab 1-1.1 as described in Materials and Methods. Lanes *A'* and *B'* are the Ab 1-1.1 immunoblots corresponding to the Coomassie-stained gels in lanes *A* and *B*, respectively. The alpha- and beta-subunits of tubulin are indicated, as well as the molecular weight (in kilodaltons) of several protein standards.

To examine if radiolabeled Ab 1-1.1 bound specifically, ME cytoskeletons were incubated with dilutions of purified ^{3}H-Ab 1-1.1 alone or in the presence of at least a 100-fold excess of unlabeled antibody from ascites serum of a hybridoma 1-1.1 tumor to determine total and nonspecific binding, respectively (Fig. 2 *A*). The level of nonspecific binding measured in the presence of excess unlabeled antibody was equivalent to binding of dilutions of the labeled antibody to culture wells without cells. Thus, it is likely that most of the nonspecific binding was to the plastic. Because excess unlabeled Ab 1-1.1 in homologous ascites serum appeared to compete with radiolabeled Ab 1-1.1 for binding to extracted ME cytoskeletons, the difference between these two sets of values in Fig. 2 *A* represented specific Ab 1-1.1 binding. As shown in Fig. 2 *B*, this specific Ab 1-1.1 binding was saturable. A Scatchard-type analysis of the data (inset) indicated that the antibody bound with a single affinity to tubulin suggesting that this type of analysis could be used to determine, by extrapolation, the total number of antigenic binding sites per cell.

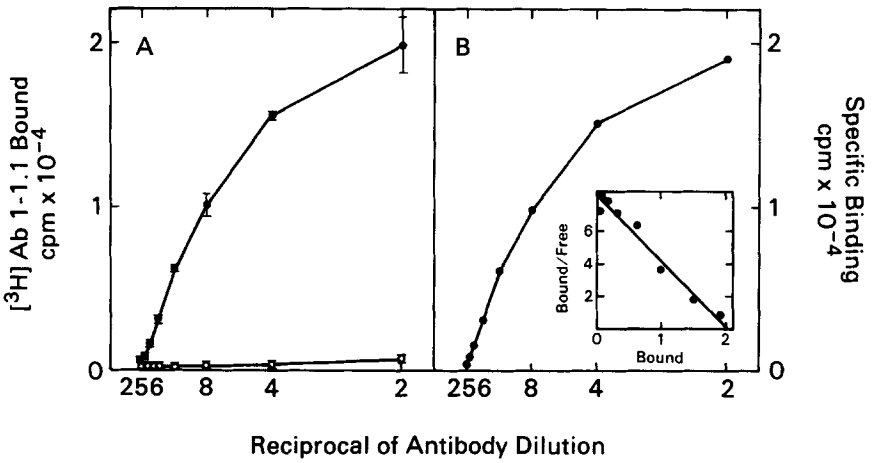

Figure 2. (*A*) Total and nonspecific binding of ^{3}H-labeled monoclonal antitubulin Ab 1-1.1 to detergent-extracted cytoskeletons of ME cells. Purified ^{3}H-labeled monoclonal Ab 1-1.1 was serially diluted as indicated and incubated overnight with formaldehyde-fixed cytoskeletons of detergent-extracted ME cells. The total radioactivity remaining bound after rinsing is shown (•). To estimate the nonspecific component of this binding, ME cytoskeletons were incubated in parallel with an identical set of labeled antibody dilutions in the presence of a large excess of unlabeled Ab 1-1.1 from an IgM fraction of ascites serum (○). Values shown are the mean ± SD of triplicate assays. (*B*) Specific binding of Ab 1-1.1 to ME cytoskeletons. Specifically bound ^{3}H-Ab 1-1.1 was calculated as the difference between the two sets of values in *A*. A Scatchard-type plot of the specific binding of antitubulin Ab 1-1.1 to extracted ME cytoskeletons is shown in the inset. Note that the specific binding of Ab 1-1.1 to the cytoskeletons is saturable and of a single apparent affinity.

Competition for Ab 1-1.1 Binding to Cytoskeletons by Nonhomologous Ascites Serum Protein or by Purified Tubulin

To determine if proteins other than the specific antibody in the ascites serum might have inhibited labeled Ab 1-1.1 binding, we compared the inhibition of binding caused by homologous and nonhomologous ascites serum (Fig. 3 *A*). As shown, as little as 20 μg of homologous ascites serum protein blocked ~60% of ^{35}S-labeled Ab 1-1.1 binding while as much as 0.7 mg of nonhomologous ascites serum protein did not compete with Ab 1-1.1 for binding to human foreskin (HF) cytoskeletons. This competition experiment demonstrated that specific Ab 1-1.1 binding was inhibited by the specific antibody in ascites fluid and not by other ascites serum proteins. It should be noted that the amount of homologous ascites serum normally added for nonspecific binding determinations in 24-well plates was 1.2 mg/well which causes maximum inhibition of Ab 1-1.1 binding.

To test if the apparent specific binding to cytoskeletons actually represented binding to tubulin, we determined if preabsorption of ^{35}S-labeled Ab 1-1.1 with purified brain tubulin could eliminate this specific component. As shown in Fig. 3 *B*, specific binding of Ab 1-1.1 was blocked by preabsorption with 9 μg/ml or more of exogenous tubulin to the same extent as by homologous ascites serum. These results together confirm that the difference between total and

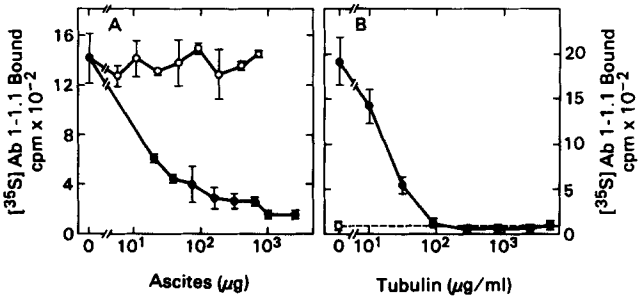

Figure 3. (*A*) Specific binding of Ab 1-1.1 is blocked by unlabeled Ab 1-1.1 ascites serum, but not by an unrelated ascites serum. Extracted cytoskeletons of HF cells were incubated with a 1:8 dilution of dialyzed culture supernatant containing ^{35}S-labeled Ab 1-1.1 in the presence of various amounts of homologous (•) or nonhomologous (○) ascites serum protein. The bound radioactivity was then determined. (*B*) Specific binding of Ab 1-1.1 is blocked by preincubation with exogenous tubulin. Aliquots of dialyzed culture supernatant fluid containing ^{35}S-labeled monoclonal Ab 1-1.1 were absorbed with the concentrations of phosphocellulose-purified bovine brain tubulin indicated before binding to HF cytoskeletons (•). A control without exogenous tubulin, but incubated in the presence of Ab 1-1.1 ascites serum is shown (○). Note that the amount of nonspecific binding was the same, whether Ab 1-1.1 binding to microtubules was competed by excess antigen (brain tubulin) or excess unlabeled antibody. Values shown are the mean ± SD of triplicate assays.

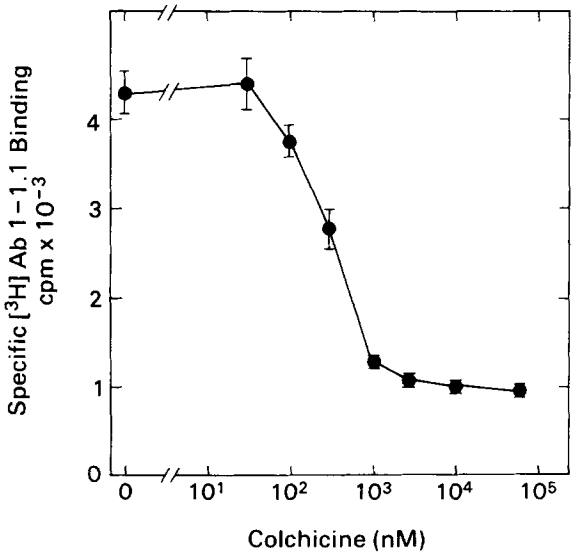

Figure 4. ^{3}H-labeled Ab 1-1.1 binding to extracted cytoskeletons of ME cells pretreated with various concentrations of colchicine. ME cells were pretreated for 1 h with the concentrations of colchicine indicated, detergent-extracted, formaldehyde-fixed, and ^{3}H-Ab 1-1.1 binding to cytoskeletons of these cells was then measured. Specific binding is shown for a single dilution (1:8) of labeled antibody. Values shown are the mean ± SD of triplicate assays.

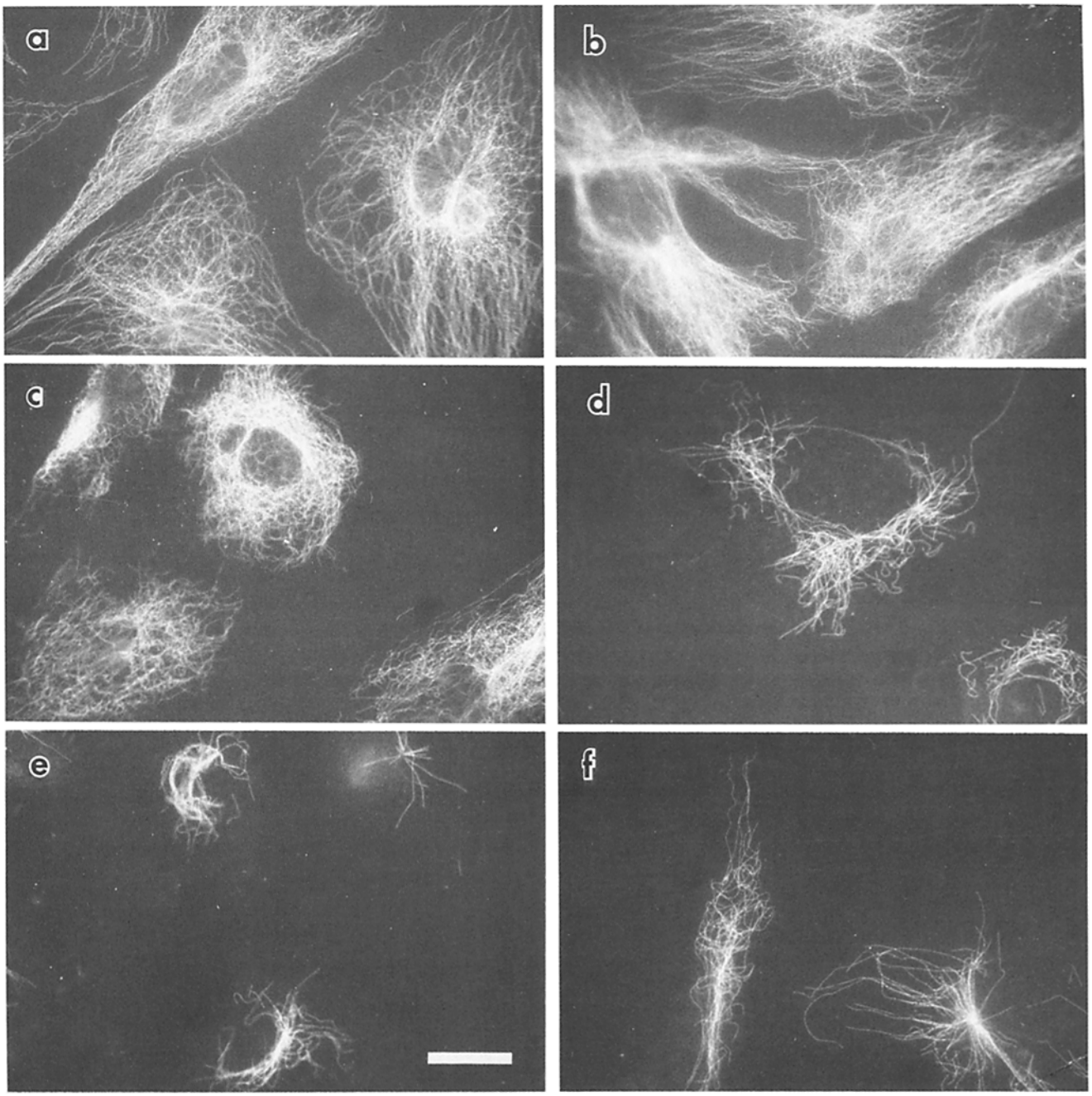

Figure 5. Antitubulin Ab 1-1.1 immunofluorescence staining of colchicine-treated ME cells. ME cells were pretreated with colchicine for 1 h before immunofluorescence analysis as described in Materials and Methods. Colchicine concentrations were as follows: (*a*) none; (*b*) 0.1 μM; (*c*) 0.3 μM; (*d*) 1 μM, (*e*) 10 μM; (*f*) 60 μM. The cells shown are representative of each population. Bar, 25 μm.

nonspecific binding in this assay represented specific binding of radioactive Ab 1-1.1 to cytoskeletal tubulin.

Binding of Ab 1-1.1 As a Function of Cytoskeletal Microtubule Content

To establish that the binding of labeled Ab 1-1.1 measured the size of the cytoskeletal tubulin pool, we assayed the Ab 1-1.1 binding to cells treated with drugs that alter microtubule content. First, ME cells were pretreated with colchicine concentrations from 0.03 to 60 μM to induce various degrees of depolymerization of cytoplasmic microtubules before the binding assay. As shown in Fig. 4, a decrease in radiolabeled Ab 1-1.1 binding was detected when ME cells were pretreated with as little as 0.1 μM colchicine and was maximal at drug concentrations from 1 to 60 μM. In various experiments, this decrease ranged from 78 to 87% of the total specific binding. Consistent with these results, parallel immunofluorescence studies showed that slight depolymerization was first seen in ME cells pretreated with 0.1 μM colchicine (Fig. 5 *b*), partial depolymerization was evident in cells pretreated with 0.3 μM colchicine (Fig. 5 *c*), and maximum depolymerization was observed in cells pretreated with 1–60 μM colchicine

Table I. Effect of Colchicine on ME Cell Attachment

Colchicine concentration	[^{3}H]Thymidine per well	% of control
μM	$cpm \times 10^{-3}$	
None	144 ± 6	100
0.03	146 ± 6	101
0.1	142 ± 14	99
0.3	158 ± 5	110
1	140 ± 2	97
3	134 ± 1	93
10	154 ± 5	107
60	130 ± 11	90

ME cells were grown in a 24-well plate in medium containing [^{3}H]thymidine for 48 h to uniformly label the cell population. Cells were then brought to quiescence as in Materials and Methods and incubated with the colchicine concentrations indicated for 1 h at 37°C. The cells were extracted and fixed as in antibody binding assays. The [^{3}H]thymidine-labeled DNA was removed from each well with 1.0 N NaOH and the radioactivity determined by liquid scintillation counting.

(Fig. 5, *d–f*). It should be noted that even at the highest colchicine concentrations these cells appear to have a population of drug-stable microtubules (Fig. 5, *d–f*) that may account for some of the residual (13–22%) specific binding of Ab 1-1.1 to these colchicine-treated cells. Other experiments showed that colchicine pretreatment, extraction, and fixation did not cause release of the cells from the assay plate (Table I). In addition, Scatchard-type analysis of the binding of Ab 1-1.1 (as shown in Fig. 2) to cytoskeletons of control ME cells and of ME cells pretreated with 1 μM colchicine demonstrated that the decrease in Ab 1-1.1 binding was due to a decrease in the number of antigenic binding sites rather than to a change in affinity (data not shown). Thus, the drug-induced decrease in specific Ab 1-1.1 binding appears to reflect an actual decrease in the amount of polymerized tubulin. Therefore, these results demonstrate that most of the specific binding of Ab 1-1.1 represents binding to microtubular tubulin.

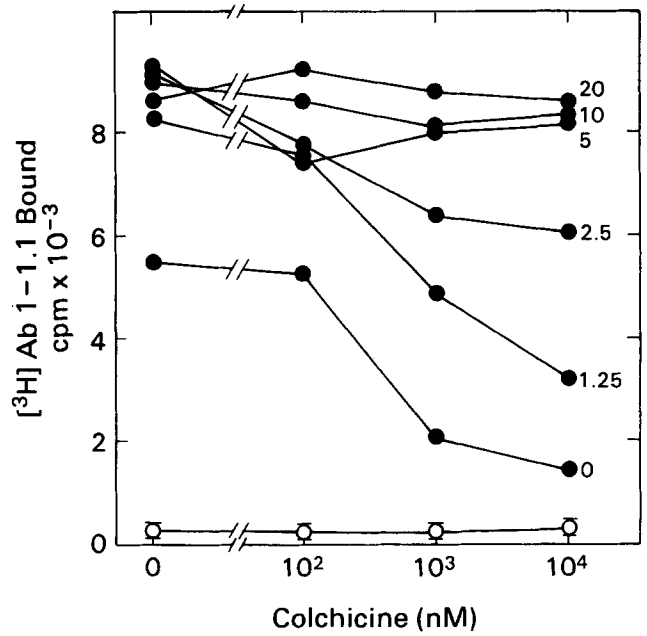

Figure 6. ^{3}H-labeled Ab 1-1.1 binding to extracted cytoskeletons of ME cells pretreated with various concentrations of taxol and colchicine in combination. ME cells were preincubated for 1 h with the concentrations of colchicine indicated on the abscissa and with the concentrations of taxol (μg/ml) indicated to the right of each curve. The cells were then extracted, fixed, and radioactive Ab 1-1.1 binding was measured in triplicate assays. Total (●) and nonspecific (○) binding of ^{3}H-Ab 1-1.1 is shown for a single labeled antibody dilution (1:8).

If the decrease in radioactive Ab 1-1.1 binding in colchicine-treated cells is due to a loss of microtubules, it should be possible to block this decrease by stabilizing microtubules with taxol. We, therefore, pretreated ME cells with various concentrations of taxol and colchicine in combination before measuring Ab 1-1.1 binding. As shown in Fig. 6, taxol treatment alone caused an increase in Ab 1-1.1 binding of ~50%. Taxol treatment of ME cells also prevented the colchicine-induced decrease in ^{3}H-Ab 1-1.1 binding to cytoskeletons in a manner dependent on taxol concentration. These results correlate with immunofluorescence studies (9) of ME cells treated with 1 μM colchicine together with various taxol concentrations. Taxol concentrations of <5 μg/ml stabilized few microtubules against disruption by 1 μM colchicine. In contrast, 5 μg/ml of taxol caused substantial stabilization and 10–20 μg/ml of taxol prevented microtubule disruption by 1 μM colchicine. Therefore, these results further indicate that radioactive Ab 1-1.1 binding is proportional to changes in cytoskeletal tubulin.

In addition to measuring drug-induced loss of microtubules, we tested the ability of this assay to measure regrowth of microtubules. We treated ME cells with colcemid and used the radiolabeled Ab 1-1.1 binding assay to monitor microtubule reassembly after removal of the drug. ME cells were treated with 1 μM colcemid for 2 h, the cells returned to fresh serum-free medium, and ^{3}H-Ab 1-1.1 binding was measured at various times after colcemid removal. As shown in Fig. 7, Ab 1-1.1 binding returned to control levels by 2 h after colcemid removal. Consistent with these results, our antitubulin immunofluorescence experiments (Crossin, K. L., and D. H. Carney, manuscript in preparation) with ME cells have shown that microtubule reassembly after removal of

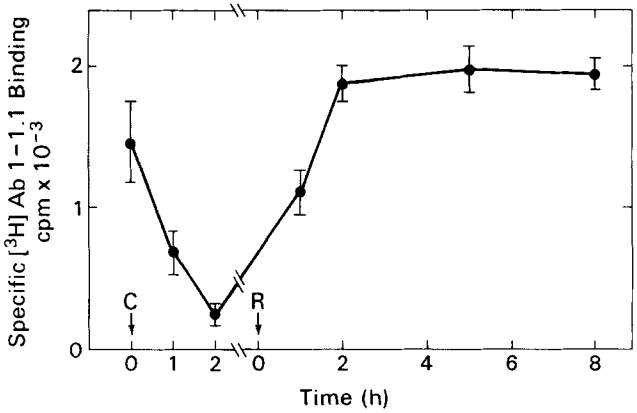

Figure 7. Effect of colcemid treatment and removal on ^{3}H-labeled Ab 1-1.1 binding to ME cytoskeletons. ME cells were treated with 2 μM colcemid (*C*) for 1 h, 2 h, or left untreated. Alternatively, the cells were treated with 2 μM colcemid for 2 h, the medium containing the colcemid removed (*R*), and the cells incubated in serum-free medium for the times indicated before binding analysis. Specific binding for a 1:8 antibody dilution is plotted. Values shown are the mean ± SD of triplicate assays.

Table II. Quantitation of the Number of ^{3}H-Ab 1-1.1 Molecules Bound to Cytoskeletons of Different Cell Types

Cell type	Antibody 1-1.1 molecules bound* (per cell)‡
ME	16×10^6
HF	10×10^6
3T3	6.1×10^6

The procedure for ^{3}H-Ab 1-1.1 binding assays and Scatchard-type analysis was described in the legend to Fig. 2.

* The specific activity of purified ^{3}H-Ab 1-1.1 was determined as described in Materials and Methods.

‡ The total number of cells per 24-well plate after extraction and fixation was determined by counting nuclei within a Zeiss ocular grid from six fields. The ocular grid was calibrated with a stage micrometer (American Optical, Buffalo, NY).

colcemid appears complete within 2 h after the drug is removed. Thus, in three different types of experiments where microtubule content was manipulated by various drug treatments, we observed a correlation between Ab 1-1.1 binding and the extent of microtubule polymerization.

Quantitation of Cytoskeletal Tubulin in Cultured Cells

The results presented above show that the specific binding of radioactive antitubulin antibodies to cytoskeletons extracted under microtubule-stabilizing conditions can be used to measure relative changes in cytoskeletal tubulin content in cultured cells. Using Scatchard-type analysis of radioactive Ab 1-1.1 binding, it is possible to determine the total number of antibody molecules that can bind per cytoskeleton. Depending on the stoichiometry of antibody binding, this number can then be used as a measure of the number of tubulin dimers present as microtubular tubulin. To test this approach to quantitation we determined the specific activity of purified ^{3}H-Ab 1-1.1 and the number of cells per multiwell plate well, and performed Scatchard-type analysis of Ab 1-1.1 binding to ME, HF, and mouse 3T3 cells (Table II). These results show that ME cells bind ~2.6 times the number of Ab 1-1.1 molecules as mouse 3T3 cells and ~1.6 times the number as HF cells. Assuming a constant ratio of bound antibody to cytoskeletal tubulin, the results in Table II indicate that these fibroblastic cell populations differ as much as 2.6-fold in their content of cytoskeletal tubulin. Immunofluorescence micrographs of these cells demonstrate that the differences in antibody binding could reflect differences in cell size (compare ME and 3T3, Fig. 8, *a* and *c*) or apparent differences in microtubule number or density (compare ME and HF, Fig. 8, *a* and *b*). It should be noted that similar results were obtained with two separate preparations of ^{3}H-Ab 1-1.1.

Hiller and Weber (16), using a radioimmunoassay for tubulin, report extraction of 26 million tubulin dimers per normal 3T3 cell and suggest that 40% of this cellular tubulin, or ~10 million dimers, would be polymerized into microtubules during interphase. We calculate that each cytoskeleton of our quiescent 3T3 cells binds ~6.1 million Ab 1-1.1 molecules at saturation. By comparing these values for 3T3 cells, we estimate that the stoichiometry of Ab 1-1.1 binding in our system is ~1.6 cytoskeletal tubulin heterodimers per IgM molecule. It should be noted, however, that our cells were growth-arrested in serum-free medium and, therefore, direct comparison with published values for nonarrested 3T3 cells may be premature. Nevertheless, the close agreement between this published value for cytoskeletal tubulin and our determination of maximal Ab 1-1.1 binding suggests that with known binding stoichiometry, this direct antibody binding assay may be quite useful in quantitating absolute amounts of cytoskeletal tubulin in cultured cells.

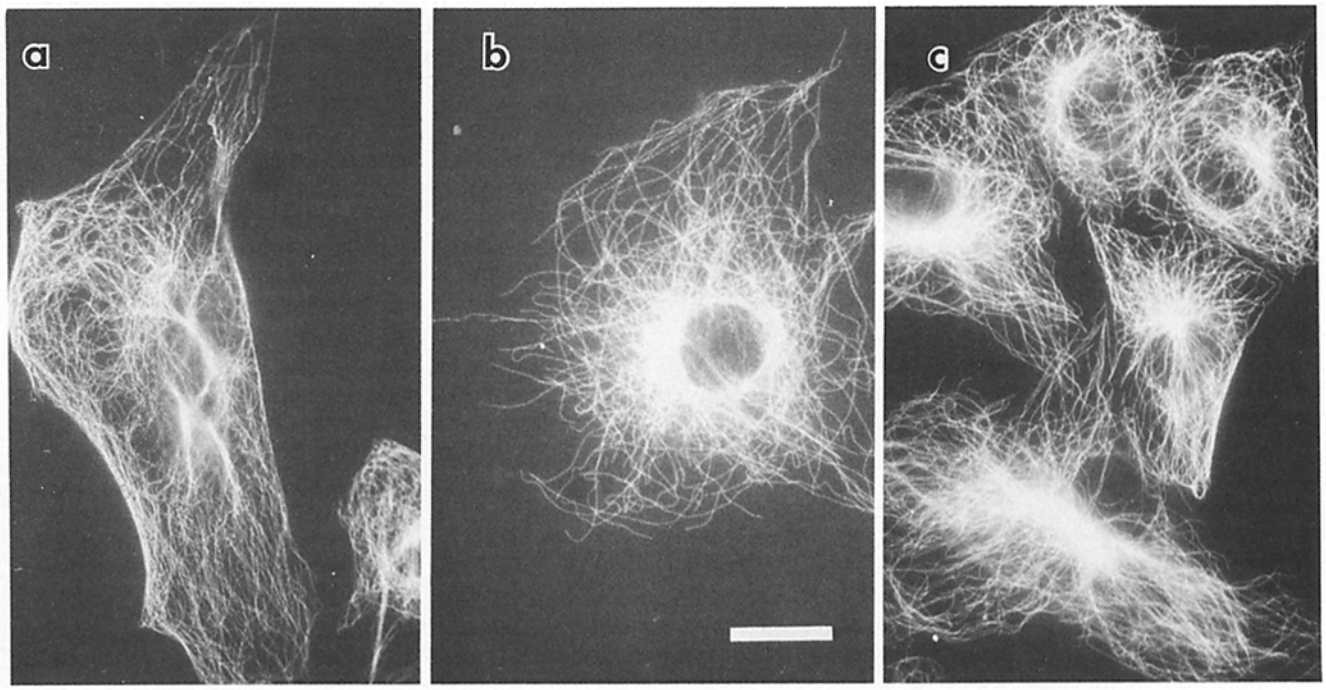

Figure 8. Ab 1-1.1 immunofluorescence staining of ME, HF, and mouse 3T3 cells. Immunofluorescence analysis was as described in Materials and Methods. (*a*) ME; (*b*) HF; (*c*) mouse 3T3. Bar, 25 μm.

Discussion

We have developed a simple, direct antibody binding assay for cytoskeletal tubulin in populations of cultured cells. This assay is based upon: (*a*) the ability to differentially extract the pool of free tubulin from the cells, leaving the microtubules with the insoluble cytoskeleton, and (*b*) the use of a monoclonal antibody to a tubulin subunit, whose saturation level of specific binding to the cytoskeletons should be directly related to the number of tubulin units in the microtubules.

Monoclonal antibody (Ab 1-1.1), specific for the beta subunit of tubulin, was translationally radiolabeled and used as a probe for cytoskeletal tubulin within detergent-extracted cytoskeletons. Specific binding of radiolabeled Ab 1-1.1 was saturable and of a single affinity. Several types of experiments indicated that this specific binding was proportional to the amount of microtubule protein within the cytoskeleton. First, pretreatment of ME cells with colchicine decreased the amount of antibody bound to cytoskeletons. This decrease correlated with the loss of microtubules as detected by immunofluorescence microscopy. Second, the colchicine-induced decrease in antibody binding could be blocked by pretreating ME cells with the microtubule-stabilizing drug, taxol, in combination with colchicine. The taxol concentrations that blocked the colchicine-induced decrease in Ab 1-1.1 binding have been previously shown to stabilize microtubules against disruption by colchicine as visualized by immunofluorescence microscopy (9). In addition, taxol treatment alone caused an increase in Ab 1-1.1 binding. Third, the radiolabeled Ab 1-1.1 binding assay was used to detect microtubule disassembly induced by colcemid, and to monitor the time course of recovery of microtubules after removal of the drug. Microtubule recovery was complete by ~2 h after colcemid removal as determined by Ab 1-1.1 binding or immunofluorescence analysis (Crossin, K. L., and D. H. Carney, manuscript in preparation). Fourth, we demonstrated with the Ab 1-1.1 binding assay that three different cell types, ME cells, HF cells, and mouse 3T3 cells, differed from each other in the amount of cytoskeletal tubulin present per cell. These differences also correlated with differences among the three cell types in the appearance of the cytoplasmic microtubule complex observed by immunofluorescence microscopy. Thus, in all cases, changes in the amount of radiolabeled Ab 1-1.1 binding reflected changes in the amount of microtubular tubulin in these cytoskeletons.

A direct radiolabeled antibody binding procedure for assaying microtubule protein has a number of advantages for estimating changes in cytoskeletal tubulin content within cultured cells. In previous direct antibody binding assays, cytoskeletal antigens have been visualized in individual cells by either immunofluorescence or immunocytochemical staining. This new assay allows quantification of changes in the polymerization state of tubulin in whole populations of cells, and does not require that cells be grown in sparse culture or that they have a flat morphology in culture. Moreover, the use of multiwell culture plates allows an entire experiment to be carried out under identical culture, binding, and rinsing conditions.

A different type of direct assay for polymerized tubulin within cultured cells uses the binding of radiolabeled taxol, a drug that binds to intact microtubules (27, 28). Although this is a promising approach to quantitating cytoskeletal tubulin, functional disadvantages of this assay procedure include the scarcity of taxol and the necessity to custom radiolabel the drug and then characterize each batch of labeled taxol for suitability in binding assays. In addition, taxol has been shown to bind preferentially to certain types of microtubules (29). Our assay shares with the taxol assay the advantage of direct binding to cytoskeletal microtubules, but has the advantage of potentially unlimited availability of both radiolabeled and unlabeled forms of reagent. The assay procedure described here for cytoskeletal tubulin should be directly adaptable for any other cytoskeletal antigen for which monoclonal antibodies are available or can be generated. This should be particularly useful for assaying proteins for which no specific drugs comparable to colchicine or taxol exist.

Differential extraction procedures have been developed for the indirect determination of microtubular tubulin content as the difference between the "unpolymerized" soluble tubulin pool and a total pool consisting of all tubulin that can be made soluble under microtubule-destabilizing conditions. Determination of the soluble tubulin in such indirect procedures has been accomplished by colchicine binding assays (3, 4, 22, 25, 26, 30–33, 40). This determination might be improved by using immunochemical assay methods. Recently, an ELISA "sandwich" procedure has been described for quantitation of the pool of soluble tubulin from cultured myocytes (34). This ELISA assay has the advantage over colchicine binding assays of stability of the binding property, and has been shown to be linear in the range of 10–500 ng of tubulin. Conventional radioimmunoassays for tubulin have also been developed in several laboratories and used for quantitation of tubulin in cells and tissues (15, 16, 18, 19, 39). Like the ELISA assay and radioimmunoassay, our assay has the advantage over colchicine binding assays that the property being measured is not labile and, therefore, does not require large extrapolative corrections to the binding data. Moreover, as opposed to indirect assays, the fixed cytoskeletons form an immobile phase making the binding and rinsing steps for large numbers of samples quite simple. However, indirect determinations of cytoskeletal tubulin, based upon colchicine binding or immunochemical determination of soluble tubulin, will remain uniquely powerful tools in assaying whole tissue homogenates as opposed to cell monolayers in culture.

The quantitation of binding of antitubulin antibody to cytoskeletons, as presented here, appears to be proportional to the content of cytoskeletal tubulin. The number of Ab 1-1.1 specific epitopes should equal the number of tubulin heterodimers. However, several factors may prevent direct quantitation of cytoskeletal tubulin molecules at this stage of development of the antibody binding assay. Absolute quantitation would require that all the epitopes be available for antibody binding and that the stoichiometry of binding could be defined. For this purpose, radiolabeled monovalent Fab fragments from an IgG would have the advantage of easier access to the epitope as well as a defined 1:1 stoichiometry. Our monoclonal Ab 1-1.1, being an IgM antibody, is not a good candidate for production of such monovalent Fab fragments. Given the nature of a complex insoluble antigen such as the cytoskeleton, some steric hinderance to binding of even

small Fab fragments may be inevitable. However, comparison of Hiller and Weber's (16) estimate of polymerized tubulin in 3T3 cells and our value for the number of Ab 1-1.1 molecules bound by 3T3 cells suggests that the overall stoichiometry for binding in our system approaches 1.6 moles of tubulin heterodimers per mole of IgM, which may indicate that most of the microtubular tubulin is, in fact, available for quantitation by this type of assay.

We have recently demonstrated that at least two antigenic types of cytoplasmic microtubules coexist within single fibroblastic cells (37). These two microtubule classes were distinguished by immunofluorescence procedures using a unique monoclonal antibody to alpha-tubulin (Ab 1-6.1). By incorporating such discriminating antitubulin reagents into the radiolabeled antibody binding assay as described here, it should be possible with this assay to observe changes within subpopulations of microtubules (or individual isotubulins within microtubules) independent of changes in the total microtubule pool. We feel that such a radiolabeled antibody binding assay, in conjunction with immunofluorescence studies based upon monoclonal antibodies of the appropriate specificity, will prove invaluable in dissecting the functional heterogeneity of the cytoplasmic microtubule complex.

We thank Gloria Herbosa and Bill Wear for their help with the immunoblots and Diane Weigent and Rhonda Peake for typing this manuscript.

This work was supported by Department of Health and Human Services grants GM-33505, AM-25807, AI-42557, and CA-17701. R. Ball is a predoctoral fellow of the McLaughlin Foundation. D. H. Carney is the recipient of a Research Career Development Award (CA-00805) from the National Cancer Institute.

Received for publication 24 March 1986, and in revised form 9 May 1986.

References

1. Asai, D. J., C. J. Brokaw, R. C. Harmon, and L. Wilson. 1982. Monoclonal antibodies to tubulin and their effects on the movement of reactivated sea urchin spermatozoa. *Cell Motil.* 1:175–180 (Suppl.).

2. Asai, D. J., C. J. Brokaw, W. C. Thompson, and L. Wilson. 1982. Two different monoclonal antibodies to alpha tubulin inhibit the bending of reactivated sea urchin spermatozoa. *Cell Motil.* 2:599–614.

3. Baraona, E., F. Finkelman, Y. Matsuda, and C. S. Lieber. 1983. A modified colchicine-binding assay for the measurement of total and microtubule-derived tubulin in rat liver. *Anal. Biochem.* 130:302–310.

4. Beersten, W., J. N. M. Heersche, and J. E. Aubin. 1982. Free and polymerized tubulin in cultured bone cells and Chinese hamster ovary cells: the influence of cold and hormones. *J. Cell Biol.* 95:387–393.

5. Bouvet, J. P., R. Pires, and J. Pillot. 1984. A modified gel filtration technique producing an unusual exclusion volume of IgM: a simple way of preparing monoclonal IgM. *J. Immunol. Methods.* 66:299–305.

6. 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. USA.* 72:4981–4985.

7. Burnette, W. N. 1981. "Western Blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195–203.

8. Carney, D. H., K. C. Glenn, and D. D. Cunningham. 1978. Conditions which affect initiation of animal cell division by trypsin and thrombin. *J. Cell Physiol.* 95:13–22.

9. Carney, D. H., K. L. Crossin, R. Ball, G. M. Fuller, T. Albrecht, and W. C. Thompson. 1986. Changes in the extent of microtubule assembly can regulate initiation of DNA synthesis. *Ann. NY Acad. Sci.* In press.

10. Crossin, K. L., and D. H. Carney. 1981. Evidence that microtubule depolymerization early in the cell cycle is sufficient to initiate DNA synthesis. *Cell.* 23:61–71.

11. Crossin, K. L., and D. H. Carney. 1981. Microtubule stabilization by taxol inhibits initiation of DNA synthesis by thrombin and by epidermal growth factor. *Cell.* 27:341–350.

12. Dustin, P. 1984. Microtubules. Springer-Verlag, New York. 267–345.

13. Edelman, G. M., and I. Yahara. 1976. Temperature-sensitive changes in surface modulating assemblies of fibroblasts transformed by mutants of Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA.* 73:2047–2051.

14. Fuller, G. M., and B. R. Brinkley. 1976. Structure and control of assembly of cytoplasmic microtubules in normal and transformed cells. *J. Supramol. Struct.* 5:497–514.

15. Gozes, I., U. Z. Littauer, B. Geiger, and S. Fuchs. 1977. Immunochemical determination of tubulin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 73:109–114.

16. Hiller, G., and K. Weber. 1978. Radioimmunoassay for tubulin: a quantitative comparison of the tubulin content of different established tissue culture cells and tissues. *Cell.* 14:795–804.

17. Inoué, S., and H. Sato. 1967. Cell motility by labile association of molecules. The nature of mitotic spindle fibers and their role in chromosome movement. *J. Gen. Physiol.* 50:259–292 (Suppl.)

18. Joniau, M., M. DeBrabander, J. DeMey, and J. Hoebeke. 1977. Quantitative determination of tubulin by radioimmunoassay. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 78:307–312.

19. Kowit, J. D., and C. Fulton. 1974. Purification and properties of flagellar outer doublet tubulin from *Naegleria gruberi* and a radioimmunoassay for tubulin. *J. Biol. Chem.* 249:3638–3646.

20. McKeithan, T. W., and J. L. Rosenbaum. 1984. The biochemistry of microtubules. *Cell Muscle Motil.* 5:255–288.

21. Masurovsky, E. B., E. R. Peterson, S. M. Crain, and S. B. Horwitz. 1981. Microtubule arrays in taxol-treated mouse dorsal root ganglion-spinal cord cultures. *Brain Res.* 217:392–398.

22. Maurice, M., G. Feldmann, B. Bellon, and P. Druet. 1980. Increase in polymerized liver tubulin during stimulation of hepatic plasma protein secretion in the rat. *BBRC.* 97:355–363.

23. Miller, C. L., J. W. Fuseler, and B. R. Brinkley. 1977. Cytoplasmic microtubules in transformed mouse X nontransformed human cell hybrids: correlation with *in vitro* growth. *Cell.* 12:319–331.

24. Morgan, J. L., and N. W. Seeds. 1975. Tubulin constancy during morphological differentiation of mouse neuroblastoma cells. *J. Cell Biol.* 67: 136–145.

25. Olmsted, J. B. 1981. Tubulin pools in differentiating neuroblastoma cells. *J. Cell Biol.* 89:418–423.

26. Ostlund, R. E. Jr., J. T. Leung, and S. V. Hajek. 1979. Biochemical determination of tubulin-microtubule equilibrium in cultured cells. *Anal. Biochem.* 96:155–164.

27. Manfredi, J. J., J. Parness, and S. B. Horwitz. 1981. Taxol binds to cellular microtubules. *J. Cell Biol.* 91 (2, Pt. 2):330a. (Abstr.).

28. Parness, J., and S. B. Horwitz. 1981. Taxol binds to polymerized tubulin *in vitro*. *J. Cell Biol.* 91:479–487.

29. Parness, J., C. F. Asnes, and S. B. Horwitz. 1983. Taxol binds differentially to flagellar outer doublets and their reassembled microtubules. *Cell Motil.* 3:123–130.

30. Pipeleers, D. G., M. A. Pipeleers-Marichal, and D. M. Kipnis. 1977. Physiological regulation of total tubulin and polymerized tubulin in tissues. *J. Cell Biol.* 74:351–357.

31. Pipeleers, D. G., M. A. Pipeleers-Marichal, P. Sherline, and D. M. Kipnis. 1977. A sensitive method for measuring polymerized and depolymerized forms of tubulin in tissues. *J. Cell Biol.* 74:341–350.

32. Reaven, E. P., Y. Cheng, and M. D. Miller. 1977. Quantitative analysis of tubulin and microtubule compartments in isolated rat hepatocytes. *J. Cell Biol.* 75:731–742.

33. Rubin, R. W., and G. D. Weiss. 1975. Direct biochemical measurements of microtubule assembly and disassembly in Chinese hamster ovary cells. The effect of intracellular contact, cold, D_2O, and $N^6O^{2'}$-dibutyryl cyclic adenosine monophosphate. *J. Cell Biol.* 64:42–53.

34. Samuel, J. L., K. Schwartz, A. M. Lompre, C. Delcayre, F. Marotte, B. Swynghedauw, and L. Rappaport. 1983. Immunological quantitation and localization of tubulin in adult rat heart isolated myocytes. *Eur. J. Cell Biol.* 31:99–106.

35. Schiff, P. B., and S. B. Horwitz. 1980. Taxol stabilizes microtubules in mouse fibroblast cells. *Proc. Natl. Acad. Sci. USA.* 77:1561–1565.

36. Sheir-Neiss, G., M. H. Lai, and N. R. Morris. 1978. Identification of a gene for beta tubulin in *Aspergillis nidulans*. *Cell.* 15:639–647.

37. Thompson, W. C., D. J. Asai, and D. H. Carney. 1984. Heterogeneity among microtubules of the cytoplasmic microtubule complex detected by a monoclonal antibody to alpha tubulin. *J. Cell Biol.* 98:1017–1025.

38. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

39. Van De Water, L., S. D. Guttman, M. A. Gorovsky, and J. B. Olmsted. 1982. Production of antisera and radioimmunoassays for tubulin. *Methods Cell Biol.* 24:79–96.

40. Waterhouse, P. D., P. J. Anderson, and D. L. Brown. 1983. Increases in microtubule assembly and in tubulin content in mitogenically stimulated mouse splenic T lymphocytes. *Exp. Cell Res.* 144:367–376.

41. Wilson, L., J. R. Bamburg, S. B. Mizel, L. M. Grisham, and K. M. Creswell. 1974. Interaction of drugs with microtubule proteins. *Fed. Proc.* 33:158–166.