A Microtubule-associated Protein Antigen Unique to Mitotic Spindle Microtubules in PtK₁ Cells

JONATHAN G. IZANT, JAMES A. WEATHERBEE, and J. RICHARD McINTOSH
Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309, and the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545. Dr. Izant's present address is the Division of Genetics, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104.

ABSTRACT Microtubule-associated proteins (MAPs) that copurify with tubulin through multiple cycles of in vitro assembly have been implicated as regulatory factors and effectors in the in vivo activity of microtubules. As an approach to the analysis of the functions of these molecules, a collection of lymphocyte hybridoma monoclonal antibodies has been generated using MAPs from HeLa cell microtubule protein as antigen. Two of the hybridoma clones secrete IgGs that bind to distinct sites on what appears to be a 200,000-dalton polypeptide. Both immunoglobulin preparations stain interphase and mitotic apparatus microtubules in cultured human cells. One of the clones (N-384.3.10) secretes antibody that reacts only with cells of human origin, while antibody from the other hybridoma (N-285.11.2) cross-reacts with BSC and PtK₁ cells, but not with 3T3 cells. In PtK₁ cells the N-285 antigen is associated with the microtubules of the mitotic apparatus, but there is no staining of the interphase microtubule array; rather, the antibody stains an ill-defined juxtanuclear structure. Further, neither antibody stains vinblastine crystals in either human or marsupial cells at any stage of the cell cycle. N-285 antibody microinjected into living PtK₁ cells binds to the mitotic spindle, but does not cause a rapid dissolution of either mitotic or interphase microtubule structures. When injected before the onset of anaphase, however, the N-285 antibody inhibits proper chromosome partition in mitotic PtK₁ cells. N-285 antibody injected into interphase cells causes a redistribution of MAP antigen onto the microtubule network.

Microtubules purified by cell fractionation or by cycles of assembly/disassembly contain, in addition to tubulin, a heterogeneous population of accessory proteins called microtubule-associated proteins (MAPs). Many of the MAPs that have been isolated are capable of stimulating microtubule assembly in vitro (6, 26, 29, 31, 34). Immunocytochemistry has shown that MAPs from brain tissue associate with microtubules in situ (8, 9, 13, 28), consistent with the hypothesis that MAPs have tubulin modulating function(s) in living cells as well as promoting tubulin polymerization in vitro. MAP cofactors may therefore mediate the cytoskeletal and motility functions of microtubules in vivo, and this has fostered our interest in the identification and characterization of those MAPs that are involved in the formation and function of the mitotic apparatus.

Brain is commonly used for the preparation of microtubule protein because it contains a high density of microtubules competent to polymerize in vitro as well as in vivo. Brain is, however, a poor source of material for the investigation of mitotic spindle components because after neurogenesis the tissue has a low mitotic index. Thus any mitotic apparatus-specific factors would be expected to be present at low concentrations. Microtubule protein prepared from cultured cells contains a different complement of assembly MAPs than neurotubule protein prepared from brain tissue (4, 27, 30, 32, 36). As first suggested by Nagle and colleagues (27), this may indicate that some brain MAPs have neuron-specific functions. Indeed, MAPs from brain either do not appear to be associated with the microtubules of nonneuronal cells (13, 24) or are found in association with the interphase microtubule network as well as the mitotic apparatus and thus are not spindle-specific components (8). There are many polypeptides that associate with HeLa cell microtubules purified by cycles of assembly/disassembly (4, 30). Such material is potentially a plentiful source of mitotic apparatus MAPs. We are producing a catalogue of specific lymphocyte hybridoma monoclonal
antibodies against the mixture of HeLa MAPs to serve as biochemical, cytological, and pharmaceutical probes for MAP function.

This report presents observations on two of the hybridoma clones that produce IgGs directed against determinants on a high molecular weight MAP from HeLa cells. While both immunoglobulins stain cytoplasmic and mitotic microtubules, microinjection of this antibody into living PtK1 cells interferes with normal mitosis, suggesting that this MAP contributes to the formation and/or function of the mitotic spindle.

**MATERIALS AND METHODS**

**Antigen and Antibodies:** HeLa cells were grown in suspension culture in modified Eagle’s medium (MEM) supplemented with 10% fetal calf serum. Cells were collected by centrifugation, washed in 200 mM PIPES, pH 6.9, 1.0 mM MgCl₂, 1.0 mM EDTA (PME buffer) and lysed by sonication at 0°C. Microtubules were precipitated and purified by cycles of assembly/disassembly as described in detail elsewhere (30). The microtubule-associated protein (MAP) fraction was isolated by ion exchange chromatography (31).

BALB/c mice were inoculated intraperitoneally with 100 μg of HeLa MAPs in complete Freund’s adjuvant and boosted intravenously 21 d later with 50 μg of HeLa MAPs in phosphate-buffered saline (PBS). On day twenty-four, the mice were sacrificed, their spleens excised, and 1.25 × 10⁶ spleen lymphocytes were fused to 1.25 × 10⁶ SP2/0 myeloma cells as described previously (15). Hybridomas were screened by a solid phase radioimmunoassay using the original HeLa MAP antigen as ligand, and antibody reacting with the immunogen was detected with 125I-Phalloidin and protein A (SAP) labeled with diiodinated Bolton-Hunter reagent (New England Nuclear, Boston, MA). Two of the hybrids (N-2B5 and N-3B4.3) that secreted antibody that bound to the HeLa MAP mixture in solid phase radioimmunoassay and stained HeLa cell microtubules by immunofluorescence microscopy were cloned twice for use in subsequent experiments. Antibritus antiserum was the generous gift of Dr. Joanna Olmsted (University of Rochester).

**Immunochernical Characterization:** The specificity of the antibodies from the resultant clones was tested by staining gel slices containing electrophoretically separated cells with proteins labeled by radiolabeled Bolton-Hunter lectin (7). HeLa MAP antigen was prepared by growing the hybridoma overnight in MEM lacking methionine (Flow Laboratories, Rockville, MD) supplemented with pyruvate, glutamine, nonessential amino acids ( Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), and 10 μg/ml of [35S]methionine (New England Nuclear). Labeled antibody was harvested by ammonium sulfate precipitation and purified by chromatography on SAP-Sepharose A-50 (Pharmacia, Piscataway, N J). A generous gift of Dr. Susan Strome (University of Colorado). They had been grown in modified Eagle’s medium (Yellow Springs Instrument Co., Yellow Springs, OH). Cells were infected with labeled monoclonal antibody bound to Sepharose by an hydroxysuccinimide linkage. An excess of monoclonal antibody to bound Sepharose by an hydroxysuccinimide linkage. An excess of monoclonal antibody to 5×10⁶ HeLa MAPs in phosphate-buffered saline was incubated with 10 mg/ml bovine serum albumin (BSA) and 10 mg/ml ovalbumin and then washed three times in PBS/O. 0.035 ml aliquots of a 50% slurry of BSA/PBS/O were added to 0.1 ml of PBS/O containing 4.0 μg of HeLa MAP antigen and 10⁵ cpm complementary 35S-labeled monoclonal antibody. In control samples the antigen was omitted or excess unbound antibody was added to assess the degree of nonspecific adsorption of radioactive label to the beads. The resin was then washed five times in PBS/O and counted in a liquid scintillation counter.

**Immunofluorescence Microscopy:** Human (Va90 and IMR), African green monkey kidney (BSC), and rat kangaroo (PtK) cells were grown in Ham’s F-12 (Gibco Laboratories) supplemented with 10% fetal calf serum. Mouse 3T3 and human HeLa cells were grown in modified Eagle’s medium (Gibco Laboratories) with 1% nonessential amino acids supplemented with 10% fetal calf and 10% calf serum, respectively. Cells were subcultured for immunofluorescence microscopy on 12-mm polylysine-coated coverslips. Cells were fixed in paraformaldehyde/glutaraldehyde as previously described (14) or, alternatively, the cells were fixed and permeabilized directly in anhydrous methanol at -20°C for 4 min followed by postfixation in acetone at -20°C for 1 min. After preincubation in 50% goat serum, the coverslips were incubated in a 1:25 dilution of primary mouse antibody in goat serum for 60 min at 37°C. After three 10-min washes in PBS the cells were incubated in secondary tetramethylrhodamine-labeled goat-antimouse immunoglobulin (U. S. Biochemicals) diluted 1:20 in goat serum. After another set of PBS washes, the coverslips were mounted in polyvinylalcohol for observation.

For some studies, primary antibody was absorbed with an excess of protein at 37°C for 4 h, 0.05-ml aliquots of a 50% slurry of PBS/O were added to 0.1 ml at 15 min for 4°C. The antibody-stained cell preparations were observed on a Zeiss microscope III equipped with epi-fluorescence optics. Images were recorded on Plus-X film and developed in HC-110.

**Microinjection:** For microinjection experiments the PtK, cells, were subcultured on 12-mm glass coverslips for 2 d, and mounted in 35-nm diameter observation chambers with 3 ml of warm medium. During prolonged observation fresh 59% O₂-equilibrated medium was circulated through the chamber at the rate of 10 ml/ h. Temperature was maintained with an air current incubator (Sage Instruments, Cambridge, MA) and monitored with a needle probe thermometer placed directly in the culture medium (Yellow Springs Instrument Co., Yellow Springs, OH). Cells were injected with microinjected antibodies (W-P Instruments, Inc., New Haven, CT) with an outside tip diameter of 0.25 μm (as determined by scanning electron microscopy) pulled on a P-77 Brown-Flaming microtip puller (Sutter Instruments, San Francisco, CA). Needles were backfilled by capillary action and then inserted into the microtubot of a Leitz micromanipulator (E. Leitz, Inc., Rockleigh, NJ).

Monoclonal antibody at a concentration of 1.0–2.5 mg/ml containing 0.5 mg/ml of fluorescein-isothiocyanate-labeled BSA was dialyzed into the injection buffer (100 mM Glutamic-Acid, 0.1 M NaOH, 1.0 mM MgCl₂, 0.1 mM dithiothreitol, pH 7.0) before use. Antibody was injected by initiating a gentle flow of antibody using air pressure developed by a 50-ml disposable syringe connected to the needle by silastic tubing and then briefly touching the needle to the cell. Injection volume was 1.4 × 10⁻¹ⁱ ml as determined by scanning electron microscopy or pulled on a P-77 Brown-Flaming micropipet puller (Sutter Instruments, San Francisco, CA). Needles were backfilled by capillary action and then inserted into the microtubot of a Leitz micromanipulator (E. Leitz, Inc., Rockleigh, NJ).

**RESULTS**

The HeLa MAP antigen mixture is composed of many polypeptides. Three of the predominant polypeptides have electrophoretic mobilities corresponding to 200,000, 125,000, and 68,000 daltons as shown in Fig. 1. Hybridoma ciones N-2B5 and N-3B4 bind to the original antigen in solid phase radioimmunoassays (data not shown), and each antibody binds to a HeLa cell polypeptide with an apparent molecular weight of ~200,000 daltons (Fig. 1). No binding to rodent cell homogenates has been observed. Like the neurotubule-associated pro-
The antibodies recognize different antigenic determinants. The N-3B4 resin is able to bind 35S-labeled N-2B5 antibody in an antigen-dependent fashion and is unbound N-3B4 success-fully competes with 35S-N-2B5 for binding to the HeLa MAP antigen. The histogram on the right shows that 35S-N-2B5 antibody binds to the N-3B4 resin in an antigen-dependent fashion and is competed off with unbound antibody. This indicates that under these conditions the two antigenic determinants are on the same structure.

FIGURE 2 Competition between N-2B5 and N-3B4 for HeLa MAPs. HeLa MAP antigen was linked to CNBr filter paper disks (An) and then incubated with [35S]methionine-labeled N-2B5 antibody (left set of histograms) or with [35S]methionine-labeled N-3B4 antibody (right set of histograms). Triplicate disks were incubated with labeled antibody in the presence of excess unlabeled N-2B5 antibody (middle histogram of each set) or with excess unlabeled N-3B4 antibody (right histogram of each set). The error bars represent the standard deviation between samples. After washing, the disks were counted in a liquid scintillation counter. The addition of excess complementary antibody has no effect on the binding of the 35S-labeled IgGs to the antigen disks. As expected, excess unlabeled antibody dramatically reduces the amount of identical 35S-labeled antibody bound to the disks. This suggests that the two immunoglobulin preparations bind to distinct antigenic determinants in the HeLa MAP mixture.

FIGURE 3 Competition experiment with resin-immobilized antibody. As diagrammed on the left, unlabeled N-3B4 antibody (Y-shaped figure) was bound to dextran beads and incubated with [35S]-labeled N-2B5 antibody (rectangular figure with asterisk). The binding of the soluble [35S]methionine-labeled N-2B5 to the resin was examined in the presence of: (A) HeLa MAP antigen (represented by the connected triangle and rectangle); (B) HeLa MAP antigen and excess unbound N-3B4 antibody; and (C) in the absence of antigen. The histogram on the right shows that 35S-N-2B5 antibody binds to the N-3B4 resin in an antigen-dependent fashion and is competed off with unbound antibody. This indicates that under these conditions the two antigenic determinants are on the same structure.

In immunofluorescence microscopy both of the monoclonal antibodies stain interphase and mitotic spindle microtubules in all human lines tested (Figs. 4 and 5 and Table 1). N-3B4 stains only cells of human origin, whereas the N-2B5 antibody stains human, nonhuman primate, and marsupial cell lines. The N-2B5 antibody does not stain 3T3 cells. The immunofluorescence images of the N-2B5 antibody in PtK1 cells is especially intriguing since only the microtubules of the mitotic apparatus...
are stained (Fig. 4). During interphase the N-2B5 antibody
stains an amorphous, frequently squiggly mass at the edge of
the nucleus. It is difficult to determine if this image corresponds
to the position of the cell center. During prophase the enlarging
spindle pole asters stain brightly, with no staining of the
residual interphase microtubules. Similarly, during telophase
there is some staining of the midbody, but no staining of the
new array of microtubules forming in each daughter cell. Thus,
the N-2B5 antibody can clearly discriminate between the micro-
tubules of the mitotic apparatus and the cytoplasmic micro-
tubules. The lack of interphase microtubule staining is not due
to inadequate fixation of the interphase microtubule arrays,
since antitubulin antisera staining and the absorption experi-
ments described below demonstrate the presence of a normal

![Figure 4](image-url)
complement of intact microtubules in the interphase cells. As with antitubulin and other anti-MAP antisera (2, 10, 33), both the monoclonal anti-MAP antibodies and the antigen-antibody complex fail to stain the center of the spindle poles and the midbody of telophase cells.

To examine the fate of the N-2B5 and N-3B4 MAPs during microtubule depolymerization, dual fluorochrome labeling of MAP and tubulin antigens was performed in HeLa and PtK₁ cells treated with the microtubule inhibitors colchicine and vinblastine. Both cell lines and both MAP antibodies gave results identical to those documented in Fig. 6. Vinblastine treatment promotes the formation of crystals of microtubule fragments (1) that stain intensely with antitubulin antibody. However, there is no detectable N-2B5 or N-3B4 staining associated with the paracrystals in either HeLa or PtK₁ cells (Fig. 6), suggesting that there is little if any association of these MAPs with vinblastine-induced crystals.

As a test for the specificity of the N-2B5 antibody, diluted preparations of the N-2B5 antibody were absorbed with purified MAP2 (17), phosphocellulose-purified tubulin (34), or the original HeLa MAP antigen. While MAP2 and tubulin have no effect on the staining pattern of the N-2B5 antibody, preparations of the N-2B5 antibody absorbed with HeLa MAPs stain both interphase and mitotic spindle microtubules with unusual brilliance in PtK₁ (Fig. 7) as well as HeLa cells (data not shown). Since the N-2B5 antibody alone does not stain interphase microtubules in PtK₁ cells, the N-2B5 antibody absorbed with HeLa MAPs must be binding to fixed interphase microtubules in a MAP-dependent fashion. Consistent with this interpretation, absorption of the N-3B4 antibody with HeLa MAPs confers on this non-cross-reacting antibody the capacity to stain microtubules in PtK₁ cells (Fig. 7). The microtubule staining by either antigen-antibody complex can be eliminated by preincubating the cells in excess HeLa MAPs. These results also indicate that the paraformaldehyde/glutaraldehyde fixation protocol used preserves the interphase as well as mitotic apparatus microtubules in PtK₁ cells.

The N-2B5 antibody was microinjected into PtK₁ cells to investigate the function of this spindle-specific antigen during mitosis. Unlike most cultured cell lines, PtK₁ cells remain flat during cell division which facilitates microinjection and observation. Fluorescein-labeled BSA was included with the antibody to allow confirmation that a cell had been injected (an absolute necessity for mitotic cells) and to permit scoring of daughter cell pairs. Injection of the non-cross-reacting N-3B4 antibody provides a valuable control for the specificity of the localization and the effect of microinjected N-2B5 antibody since both immunoglobulin preparations bind to MAPs, but only the N-2B5 reacts with PtK₁ cells.

To determine whether the microinjected antibody had any effect on mitosis and cytokinesis, metaphase and prometaphase PtK₁ cells were injected with antibody and incubated 3 h before fixation and examination. Cells that appeared to have been damaged by the injection were not scored. As shown in Table II, the fraction of cells able to complete mitosis successfully within the incubation period was lowered from 70% for cells injected with the control N-3B4 antibody (which is comparable to that found when buffer alone is injected), to 14.6% for cells injected with the N-2B5 antibody. The introduction of N-2B5 immunoglobulin into living PtK₁ cells produces a distinct inhibition of normal mitosis.

Cells were also fixed at 5, 30, and 120 min after injection and stained with rhodamine-labeled rabbit anti-mouse immu-
noglobulin antibody to determine the distribution of the injected antibody. As shown in Fig. 8, after 5 min the distribution of the microinjected antibody corresponds to that seen in the fixed preparations; mitotic spindles stain brightly and in interphase cells there is perinuclear staining concentrated at a focus. After 30 min, the spindles are reduced in size and clarity; after 2 h, they often appear as a pair of stained masses. Surprisingly, 30 min after injection of interphase cells, the N-2B5 antibody stains the cytoplasmic microtubule array in approximately one-third of the cells; after 2 h up to half of the interphase cells show microtubule staining. There is no apparent disassembly of the interphase microtubule network concomitant with this redistribution of the anti-MAP antibody.

Individual cells were followed by DIC and polarization microscopy to examine the details of the mitotic inhibition. As represented in Fig. 9, most of the cells injected during metaphase or prophase do not start anaphase. There is a gradual disorganization of the chromosome arrangement in metaphase cells, though the effect is never quite so marked as is seen in colchicine- or nocodazole-treated cells. In time-lapse video recordings, the chromosomes appear to maintain some structural attachment to the spindle. Using polarization optics to assess spindle fiber structure, there is no rapid pathological effect of antibody injection on the organization of the spindle itself. Rather, there is a gradual decrease in spindle size and in the magnitude of spindle birefringence during the 10 min following injection of the N-2B5 antibody. In those cells that initiate anaphase, chromosome velocity is approximately half that seen in normal or N-3B4 injected cells, and the extent of chromosome separation is significantly less than that in normal cells. Injection of N-2B5 antibody after the start of anaphase chromosome movement has no apparent effect on the rate or extent of chromosome separation or on subsequent cytokinesis.

FIGURE 6 Dual Fluorochrome labeling of tubulin and N-2B5 antigens in colchicine and vinblastine-treated cells. PtK1 cells were treated with $10^{-9}$ M colchicine (a and b) or with $10^{-9}$ M vinblastine sulfate (c and d) for 2 h before fixation and then stained with rabbit antitubulin and mouse monoclonal N-2B5 antibody. Anti-tubulin was detected with fluorescein-conjugated goat anti-rabbit immunoglobulin and the N-2B5 antibody was detected with tetramethyl-rhodamine-conjugated goat anti-mouse immunoglobulin. Colchicine produced diffuse distributions of both antigens (a and b). Images recorded using fluorescein excitation and barrier epifluorescence filters (a and c) document the presence of tubulin paracrystals in vinblastine-treated cells. Micrographs recorded with rhodamine filters (b and d) show that both in interphase and mitotic cells the vinblastine-induced tubulin paracrystals do not contain detectable N-2B5 MAP. Bar, 10 μm. × 1,250.

DISCUSSION

The present results suggest that hybridoma clones N-2B5 and N-3B4 secrete IgGs that bind to distinct sites on a HeLa MAP of ~200,000 daltons. This MAP binds to microtubules during cycles of in vitro assembly (31) and is associated with interphase and mitotic spindle microtubules in human cells. For reasons that remain enigmatic, antibody staining of polyacrylamide
gels and nitrocellulose replicas as well as immunoprecipitation and affinity chromatography techniques with native and denatured cell homogenates have repeatedly failed to elucidate the electrophoretic mobility of the N-2B5 antigen in PtK1 cells. Regardless, the immunofluorescence and microinjection experiments indicate that the N-2B5 antibody binds to a microtubule-associated protein in PtK1 cells. Further, the N-2B5 antibody has permitted us to identify a unique spindle component in PtK1 cells and to document its participation in mitotic chromosome partition.

A rabbit antiserum to a 210,000-dalton HeLa assembly MAP has been prepared (5). Although this antiserum reacts with a HeLa MAP of a molecular weight similar to that of the N-2B5 and N-3B4 antigen, the cross-reactivity of the antiserum is different from the cross-reactivities of both N-2B5 and N-3B4. Whereas the antiserum reacts with human and several primate cell lines, the N-3B4 antibody appears to be human-specific. In contrast to both, the N-2B5 antibody shows cross-reactivity in both primates and marsupials. It is possible that the rabbit antiserum reacts with only a subset of the 200,000-dalton HeLa MAPs, and thus does not stain cells lacking those particular MAPs. Alternatively (or in addition), the N-2B5 antibody may bind a MAP antigenic determinant that is common between HeLa and PtK1 cells but that is not recognized by the antiserum.

The experiments with antigen immobilized on CNBr cellulose disks and those with antibody bound to chromatography resin both indicate that the N-2B5 and N-3B4 antibodies bind

---

**FIGURE 7** Staining of PtK1 cells with absorbed MAP antibodies. PtK1 cells were stained with N-2B5 anti-MAP antibody absorbed with HeLa MAP antigen (a and b), with N-3B4 absorbed with HeLa MAPs (c) or with N-2B5 antibody absorbed with purified MAP2 (d). The antigen-antibody complex stains both interphase and mitotic spindle microtubules, whereas absorption with MAP2 or tubulin produces images similar to the N-2B5 antibody alone. Bar, 10 μm. X 1,250.

---

**TABLE II**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cells* scored</th>
<th>Daughter pairs</th>
<th>Binucleate cells</th>
<th>Unsuccessful karyokinesis$^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-3B4.3.10</td>
<td>109</td>
<td>65 (60%)</td>
<td>11 (10%)</td>
<td>33 (30%)</td>
</tr>
<tr>
<td>N-285.11.2</td>
<td>226</td>
<td>21 (9.3%)</td>
<td>12 (5.3%)</td>
<td>193 (85%)</td>
</tr>
</tbody>
</table>

* Cells were scored 3 h after injection

$^\dagger$ Includes mitotic arrest and mononuclear cells
to distinct, nonoverlapping sites on HeLa MAPs. The ability of immobilized N-3B4 antibody to bind 35S-labeled N-2B5 antibody in an antigen-dependent fashion suggests that the two immunoglobulins bind to a single polypeptide. However, we cannot unequivocally exclude the possibility that the two antibodies recognize two distinct 200,000-dalton MAPs that bind tightly to one another. Indeed, gel filtration studies demonstrate that native high molecular weight HeLa assembly MAPs exist as dimers in solution (6). If N-3B4 and N-2B5 bind distinct polypeptides, then the current data suggest that the high molecular weight HeLa MAP exists as a heterodimer of N-2B5 and N-3B4 antigen subunits in vivo.

The observations on the coincident localization of the HeLa MAP antigens and microtubules in immunofluorescence experiments support the theory that polypeptides that copurify with tubulin in vitro also interact directly with microtubules in vivo. These observations are consistent with the hypothesis that MAPs play a direct role in the modulation of tubulin assembly and/or function in living cells. Furthermore, the current results indicate that in PtK₁ cells the N-2B5 antigen is associated specifically with the mitotic spindle. The N-2B5/PtK₁ antigen is a novel MAP in its capacity to discriminate between different classes of cytoplasmic microtubules in cultured cells. Although immunocytochemical studies have previously shown that calmodulin (35), cyclic nucleotide-dependent kinase (3), and NuMA (21) also associate specifically with mitotic spindle microtubules, no other accessory proteins from cycled brain or HeLa microtubule protein have been found in unique association with mitotic apparatus microtubules (5, 8, 9, 10, 13, 28).

Our collection of anti-MAP hybridomas includes one clone (O-2D5) that produces IgG that preferentially stains microtubules in the mitotic spindle of human cells, while in interphase cells it stains the interphase microtubules faintly and produces a distinctive punctate nuclear staining pattern (15, 16). Whereas the O-2D5 MAP antigen can be sparsely detected along interphase microtubules, the N-2B5 MAP antigen associates exclusively with mitotic spindle microtubules in PtK₁ cells and cannot be detected on interphase PtK₁ microtubules at all. The only precedent for the interaction of an assembly-promoting MAP with a specific subset of cellular microtubules is the brain polypeptide MAP2 which has been localized to dendritic processes in situ (24).

The 200,000-dalton MAP (or MAPs) and tubulin are not always found associated with each other as evidenced by the vinblastine-induced tubulin paracrystals that do not contain detectable amounts of either the N-2B5 or N-3B4 antigen. While consistent with the in vitro results of Luduena and coworkers (20), our images are in contrast to observations made by other workers (10, 11) who found that a 210,000-dalton HeLa MAP antigen was associated with vinblastine-induced

---

**Figure 8** Distribution of microinjected N-2B5 anti-MAP antibody in PtK₁ cells. Mitotic (a and b) and interphase (c and d) PtK₁ cells were microinjected with N-2B5 antibody, incubated for 5 min (a and c) to 30 min (b and d) and then fixed and stained with fluorochrome-labeled secondary antibody. The N-2B5 antibody binds readily to mitotic spindles in living as well as fixed PtK₁ cells and promotes a gradual disorganization of the spindle. When injected into interphase cells the N-2B5 antibody promotes a redistribution of antigen onto the interphase microtubule array (d). Bar, 10 μm. X, 1,250.
tubulin aggregates. The reasons for this discrepancy are not obvious. It may be that the determinants recognized by the N-2B5 and N-3B4 antibodies are masked in the crystals, or perhaps the antiserum used in other studies contains a heterogeneous group of antibodies against several high molecular weight HeLa polypeptides, one of which does bind to vinblastine crystals.

The serendipitous absorption experiment indicates that preincubation of antibody with HeLa MAPs allows the N-2B5 and the N-3B4 immunoglobulins to bind to interphase PtK1 cell microtubules via the HeLa MAPs. The soluble antigen-antibody complex is in fact an excellent probe for microtubule distribution. The antigenic determinants recognized by the N-2B5 and the N-3B4 antibodies do not appear to be part of the microtubule binding site on the MAP molecule, since the soluble antigen-antibody complex binds readily and specifically to microtubules in situ. The results also demonstrate that chemically fixed microtubules can be specifically recognized by microtubule-associated proteins. This supports the notion that chemically stabilized microtubules are valuable affinity

---

**Figure 9** Microinjection of monoclonal anti-MAP antibodies into metaphase PtK1 cells. Cells were observed with polarization (a) or DIC optics (b-d) after the injection of N-2B5 (a-c) or N-3B4 (d) antibodies. Time shown is minutes after antibody injection. Injection of N-2B5 antibody promotes a reduction in spindle size and birefringence (a) in PtK1 cells. Most cells do not enter anaphase (b); rather there is disorganization of the chromosome arrangement. Some PtK1 cells injected with N-2B5 antibody initiate chromosome separation (c) but it is neither as fast nor as extensive as in cells injected with the non-cross-reacting N-3B4 antibody (d). Bar, 10 μm. × 700.
ligands for the biochemical identification of cultured MAPs (14). The staining intensity of the antigen-antibody complex is actually greater than that detected with the antibody alone, suggesting that the binding of MAPs along microtubules is not at saturation in vivo. This extends the observations of Kim and colleagues (17) on neurotubules that there are significant numbers of MAP binding sites on the tubulin polymer lattice that are normally unoccupied.

Microinjection of monoclonal antibodies provides an approach to the production of phenocopies of MAP structural gene mutations in a biological system that is not readily amenable to thorough genetic analysis (19, 22). The introduction of the N-2B5 antibody into living cells provides a novel view of MAP function in vivo. As summarized in Table II, the microinjection of N-2B5 antibody seriously impairs the ability of PtK cells to complete karyokinesis successfully. Due to the intrinsic biological variability of the system and the complexity of the effects it was necessary to examine several hundred injected cells to be confident of the observations.

Immunofluorescence microscopy confirms that the N-2B5 antibody binds rapidly and specifically to the mitotic spindle in living as well as fixed PtK cells. Observations with fluorescence, polarization, and DIC optics indicate that the injection of N-2B5 promotes a slow disorganization of the mitotic apparatus rather than a rapid disassembly, as is the case with calcium ion injections (Izant, J., manuscript in preparation). Thus, the antigenic determinant recognized by the N-2B5 antibody is probably not directly involved in microtubule assembly and, as demonstrated in the antigen absorption experiments, is probably not part of the tubulin binding site.

The rate and extent of chromosome movement in the injected cells that initiate anaphase is reduced, but nevertheless chromosome movement is still possible. Thus while mitotic apparatus motors are not completely jammed, the N-2B5 antibody may inhibit microtubule elongation in metaphase cells or act as a "monkey wrench" for a subset of microtubule interactions. The lack of effect when N-2B5 antibody is injected into anaphase cells is potentially due to the lag period necessary for antigen-antibody binding, but it may further suggest that the N-2B5 antigenic determinant in PtK cells is not the only site of mitotic force production.

Since the N-2B5 MAP antigen in PtK cells clearly differentiates between interphase microtubule networks and mitotic spindle fibers, it would be valuable to determine whether the discrimination arises from the MAP binding to some other spindle-specific factor that is in turn bound to spindle microtubules or whether it is due to a cell cycle-dependent change in the PtK N-2B5 MAP antigen that promotes microtubule binding only during mitosis. The current results favor the latter possibility. The redistribution of microinjected N-2B5 antibody onto microtubules in interphase cells is probably a reflection of the redistribution of the PtK MAP itself, since, even when high concentration of the antibody are incubated with fixed PtK cells for long periods, the N-2B5 antibody does not detect any antigen on fixed interphase microtubules. The antibody-induced redistribution of the PtK MAP following microinjection demonstrates that this MAP can bind to microtubules during interphase and suggests that it is a chemical or conformational change in the N-2B5 MAP or MAP complex that promotes microtubule binding during mitosis. This binding may be physiologically significant in modulating mitotic apparatus formation in vivo.

We are grateful to Michael Zavortink for patient instruction on microinjection techniques and to Lynn Van Meter and Cathy Inouye for their frequent typing of the manuscript.

This work was supported by grant CD-8 from the American Cancer Society; J. Izant was supported in part by a postdoctoral Fellowship from Training Grant #5T32HD07070 from The National Institutes of Health to Keith R. Porter. The microinjection equipment was purchased with Biomedical Research Support Grant RR07103-14 to the University of Colorado from the Division of Research Resources, National Institutes of Health.

Received for publication 22 February 1982, and in revised form 18 October 1982.

REFERENCES