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Microtubule-associated proteins: A monoclonal antibody to MAP2 binds to differentiated neurons

(microtubule assembly/tubulin/high molecular weight component/lymphocyte hybridoma/neuronal development)

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ABSTRACT **Hybridomas that secret IgG reacting specifically with the brain microtubule-associated protein MAP2 have been prepared with spleen cells from BALB/c mice hyperimmunized with high molecular weight neurotubule-associated proteins. Immunofluorescence microscopy using dual fluorochrome labeling of tubulin and MAP2 antigens revealed identical patterns of interphase fiber networks in cells from explants of newborn mouse brain. The anti-MAP2 antibody did not stain primary mouse kidney cells or CHO, 3T3, HeLa, or PtK1 cell lines. Immunoprecipitation and antibody gel staining techniques failed to demonstrate any crossreacting antigen in these cells. MAP2 antigen was not seen in association with the mitotic spindle in any of the cells examined. Radioimmunoassay showed species crossreactivity of the anti-MAP2 antibody with mammalian but not avian neural cell extracts. Glial cells and some neuroblastoma cell lines did not appear to contain MAP2. However, in the B104 rat neuroblastoma cell line the MAP2 antigen appeared to be associated with the cytoskeleton concomitant with differentiation induced by dibutyryl cyclic AMP. In disagreement with most previously published reports, our data suggest that MAP2 is found only in differentiated neuronal cells and raises the possibility that MAP2 is involved in neuronal differentiation or neuron-specific processes.**

Two classes of microtubule-associated proteins (MAPs) copurify with brain tubulin through cycles of assembly/disassembly: tau factor, 55,000–62,000 daltons (1, 2); and the high molecular weight protein (HMW), 250,000–350,000 daltons (3, 4). These MAPs increase rates of both nucleation and elongation during tubulin polymerization *in vitro* (2, 4), and it has been suggested that they play a role in the control of the *in vivo* utilization of microtubules during interphase and cell division. Consistent with this hypothesis, antisera prepared against MAPs stain interphase microtubule networks and mitotic spindles in a wide variety of cells (5–9). Recently, however, carefully characterized antisera to high molecular weight assembly MAPs from brain and cultured cells have been shown to have limited crossreactivity between different cell types (ref. 10; unpublished results) and species (11).

The reasons for the discrepancy between the different antisera are not easily understood. Serum contains many different immunoglobulins, and it is possible that one subset of immunoglobulin is giving rise to immunofluorescent staining patterns whereas another is responsible for Ouchterlony immunoprecipitation lines. This complexity is not remedied by affinity chromatography purification of the antiserum because, during elution, partial denaturation of antibody may occur, possibly modifying the specificity of the immunoglobulins.

To alleviate some of the problems inherent in the use of animal sera, we have been studying the *in vivo* function of HMW by using monoclonal antibody probes produced against MAPs according to the lymphocyte hybridoma method originated by Kohler and Milstein (12). In this report we present evidence that the lower molecular weight HMW protein, MAP2 (13), is found only in differentiated neuronal cells in a wide variety of mammals. Our monoclonal antibody to MAP2 does not stain the mitotic apparatus or cytoplasmic microtubules in nonneuronal tissue or cell lines. Examination of neuroblastoma cell lines which differentiate in culture suggests that the appearance of MAP2 may be associated with neuronal differentiation both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Antigen Preparation. Microtubule protein (MTP) was prepared from the brain of freshly slaughtered hogs by two cycles of assembly/disassembly (3) and stored as pellets at −70°C. MAP2 was prepared by the method of Kim *et al.* (13). HMW antigen was prepared by separation of MAPS from MTP on a phosphocellulose column (3) and electrophoresis of the MAPs on $NaDodSO_4$/polyacrylamide slab gels (2.5 mm thick and 100 mm wide, 6% acrylamide) (14). The bands containing HMW were excised and the protein was extracted by electroelution and acetone precipitation. Protein assays were done by the method of Bradford (15).

Hybridoma Fusion. Female BALB/c mice were injected intraperitoneally with 100 μg of HMW in Freund's complete adjuvant (Difco) and boosted intravenously 21 and 24 days later with 100 μg of HMW in Earle's phosphate-buffered saline without calcium (P_i/NaCl). On day 27, two mice were sacrificed and their spleens were excised; 1.65×10^8 spleen cells were fused to 1.65×10^7 logarithmic phase SP2/0 mouse myeloma cells in 30% (wt/vol) polyethylene glycol 1000 by using modifications of the methods of Gefter *et al.* (16) and Claflin (17). Fourteen days after fusion, samples of the cell supernatants were screened for the production of specific antibody, and the cells from positive wells were cloned twice in RPMI-1640 containing 10% heat-inactivated fetal calf serum, 8% heat-inactivated horse serum (GIBCO), and 0.5% agarose (Seaplaque, Marine Colloids). Antibody-producing hybrids were grown in RPMI-1640 supplemented with 5% NCTC 109 medium and 10% heat-inactivated fetal calf serum (R/N.10) in roller bottles or as ascites tumors in Pristane-primed BALB/c mice. Antibody produced in tissue culture was concentrated 50-fold before storage. Rabbit serum raised against tubulin was the generous gift of J. Olmsted.

Solid-Phase Assay. Four-day cell supernatants were assayed in 60-well Terasaki plates (Falcon 3034). A 0.2- to 2-μg sample of antigen was dried in each well or, alternatively, the plates were pretreated with 1.0% 1-ethyl-3(dimethylaminopropyl)-carbodiimide (Sigma) and thoroughly rinsed with P_i/NaCl before the addition of antigen. The plates were washed twice with P_i/NaCl and once for 10 min with P_i/NaCl containing ovalbumin (1 mg/ml). The pretreated plates were washed for

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Abbreviations: MAP, microtubule-associated protein; HMW, high molecular weight protein; MTP, microtubule protein; P_i/NaCl, Earle's phosphate buffered saline without calcium.

10 min with 0.1 M Tris (pH 7.4) containing ovalbumin (1 mg/ml). After two more rinses with P_i/NaCl, 15 μl of supernatant to be tested was added to each well and the plates were incubated at 37°C for 1 hr. After two rinses with P_i/NaCl, one 10-min wash with P_i/NaCl plus ovalbumin, and two more rinses with P_i/NaCl, all at 0–4°C, approximately 50,000 cpm of ^{125}I-labeled *Staphylococcus* protein A (Pharmacia) labeled to a specific activity of 20 mCi/mg (1 Ci = 3.7×10^{10} becquerels) by the chloramine-T method (18) was added to each well. After a 15-min incubation at room temperature the plates were washed as before with P_i/NaCl at 0–4°C, washed with cold distilled water, dried, and autoradiographed with X-Omat RP film (Kodak) and a Cronex intensifying screen (DuPont) at −70°C for 6–18 hr.

Antibody Specificity. The specificity of the monoclonal antibody preparations was determined by staining of NaDodSO$_4$ gels and radioimmunoassay. Antigens were detected in slices of 7.5% NaDodSO$_4$/polyacrylamide gels fixed in 15% trichloroacetic acid and methanol/acetic acid by the method of Burridge (19). Iodinated *Staphylococcus* protein A was used to probe for bound IgG. Tritiated monoclonal antibody was obtained by growing the IgG-secreting hybridoma cells in medium containing [^{3}H]leucine (New England Nuclear) at 0.5 μCi/ml. For radioimmune determination of the presence of antigen, tissue and cell samples were homogenized or sonicated in 4 vol of 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.95/1.0 mM $MgCl_2$/1.0 mM ethylene glycol bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA)/0.1% Aprotinin (Sigma)/0.1% phenylmethylsulfonyl fluoride, extracted on ice for 1 hr, and clarified at 8000 × *g* for 10 min at 4°C. These samples, diluted to various extents in P_i/NaCl, were then incubated with 10,000–20,000 cpm of tritiated antibody for 30 min at 37°C. Purified antigen covalently bound to Sepharose beads (Affi-Gel 10, Bio-Rad; 0.1 ml, 25 μg of antigen per sample) was added, and the mixture was incubated an additional 60 min at 37°C with shaking. The antigen beads were then washed with P_i/NaCl four times by centrifugation, including a wash with the P_i/NaCl/ovalbumin mixture, and an aliquot of the bound immunoglobulin was assayed in a scintillation counter.

Indirect Immunofluorescence Microscopy. 3T3 and HeLa cells were grown in Dulbecco's minimal essential medium (GIBCO) plus 10% fetal calf serum; CHO, PtK1, and N18 cells were grown in Ham's F-12 (GIBCO) plus 10% fetal calf serum; and neuroblastoma B104 and N2a cell lines were grown in a 1:1 mixture of Ham's F-12 and Dulbecco's medium supplemented with 8% fetal calf serum and 5% horse serum. Primary cultures of mouse brain and kidney cells were grown in Dulbecco's medium containing 10% fetal calf serum and 5% horse serum. For immunofluorescence microscopy the cells were grown on 12-mm polylysine coated coverslips for 2–5 days, rinsed in warm 0.1 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.95/1.0 mM $MgCl_2$/1.0 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′ *N*′-tetraacetic acid (PME buffer), and fixed in 2% paraformaldehyde/0.1% glutaraldehyde (Ladd) in PME buffer for 20 min at 37°C. The cells were rinsed in PME, postfixed, and made permeable in acetone at −20°C for 15 min. After rinsing with distilled water, the cells were rehydrated in P_i/NaCl, preincubated in 1% goat serum (GIBCO) for 15 min, washed in P_i/NaCl, and inverted on a 25-μl drop of antibody diluted in P_i/NaCl containing 10% of the appropriate nonspecific serum. After 1 hr at 37°C in a humid chamber the coverslips were washed for 30 min in three changes of P_i/NaCl at 4°C and then inverted on a 25-μl drop of fluorochrome-conjugated antibody diluted in 10% serum. After 45 min at 37°C, the coverslips were washed as before, rinsed in distilled water, and mounted on a drop of 30% Gelutol (Monsanto)/30% glycerol in P_i/NaCl. For dual fluorochrome labeling, the order of incubation was rabbit anti-tubulin, fluorescein-conjugated goat anti-rabbit IgG (Miles), monoclonal anti-MAP2, and rhodamine B-conjugated rabbit anti-mouse IgG (Miles).

RESULTS

Fourteen days after the fusion of the myeloma and spleen cells, one to eight colonies of hybrid cells were growing per well. Solid-phase immunoassays revealed nine wells containing IgG which bound to electroeluted HMW antigen, phosphocellulose-purified MAPs, or MTP. Cells from two wells were successfully cloned. Two of the clones, D-1D1.16.6 and D-1D1.43.2, were used in subsequent experiments.

The specificity of the antibody was first examined by antibody staining of NaDodSO$_4$ gel slices. Fig. 1*a* shows the Coomassie blue staining pattern of a gel slice containing MTP and homogenates of four cultured cell lines. Fig. 1 *b* and *c* shows the autoradiographs of identical gel slices incubated with a control monoclonal antibody (B-3A3.2, an IgG-secreting hybrid from a fusion using unimmunized mice) and with D-1D1.16.6 antibody, respectively. The nonspecific binding of the ^{125}I-labeled *Staphylococcus* protein A was low, and the D-1D1.16.6 antibody bound to a single band, corresponding to the lower molecular weight HMW band, MAP2. This band reportedly runs as a doublet (4) but we could resolve only one evenly staining band, suggesting there may be some antigenic relationship between the two MAP2 bands, but MAP1 and MAP2 have at least some antigenically distinct domains. Within the detection efficiency of this method (see ref. 19), there appears to be no similar antigen in any of the cell lines.

Several tissue and cell line homogenates were examined for their ability to compete with MAP2 antigen bound to dextran beads for the binding of labeled antibody (Fig. 2). The results show crossreactivity among bovine, porcine, and mouse brain but not with chicken brain. MAP2 antigen does not appear to be present in liver or kidney samples from mice. Consistent with the gel localization data, the radioimmunoassay did not detect significant amounts of MAP2 antigen in any of the cultured cell lines examined.

The indirect immunofluorescence image of cells stained with our antibodies varied with the fixation protocol used. The addition of a low concentration of glutaraldehyde gave better preservation of microtubules than did paraformaldehyde or acetone alone but did not mask antigenic determinants. No

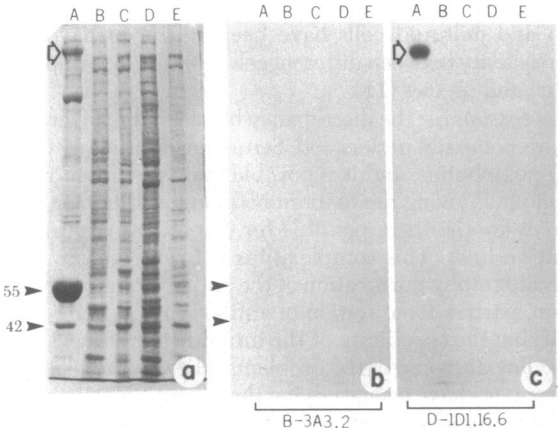

FIG. 1. Antigen detection in 7.5% NaDodSO$_4$/polyacrylamide gels. (*a*) Stained with Coomassie blue. (*b* and *c*) Autoradiographs of identical slices probed with ^{125}I-labeled *Staphylococcus* protein A after staining with control monoclonal antibody B-3C3.2 (*b*) or anti-MAP2 antibody D-1D1.16.6 (*c*). Lanes: A, MTP; B–E homogenates of HeLa, 3T3, CHO, and PtK1 cells, respectively, clarified by centrifugation at 8000 × *g* for 4 min.

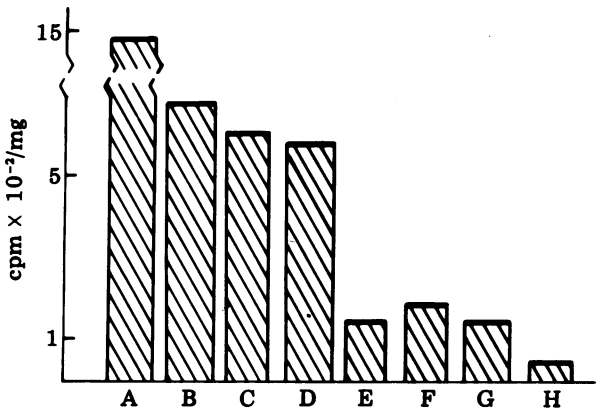

FIG. 2. Histogram showing the relative amounts of MAP2 antigen in several tissues and cell types as determined by radioimmunoassay. Ordinate represents the relative amount of ^{3}H-labeled D-1D1.16.6 antibody bound per mg of sample. Sample (100 μl containing 0.05–1 mg of protein) was incubated with 12,000 cpm of labeled antibody for 30 min. Purified MAP2 covalently bound to Sepharose was used to assay for unbound antibody. MAP2 antigen content was calculated by subtracting the cpm of antibody bound to the Sepharose beads from the total amount of available antibody and dividing by the weight (mg) of sample added. Purified MAP2 bound 94% of the labeled antibody, while the background was approximately 200 cpm. A, MAP2; B, MTP; C, hog brain; D, mouse brain; E, chicken brain; F, mouse kidney; G, mouse liver; H, HeLa cells.

postfixation reduction of unreacted aldehyde was found to be necessary. Because this fixation procedure extracted the cells less than did acetone or paraformaldehyde alone, there is some background staining, presumably due to the fixation of soluble protein.

Most primary brain cells examined with the D-1D1.16.6 antibody revealed complex patterns of fiber bundles and delicate fiber networks (Fig. 3), similar to those observed with anti-tubulin (20). Treatment with the microtubule inhibitor nocodazole (Fig. 3*c*) or cold eliminated the fibers, which resulted in diffuse staining patterns with both anti-tubulin and anti-MAP2 antibody. Preabsorption of anti-MAP2 antibody with electroeluted HMW, phosphocellulose-purified MAPs, or microtubule protein eliminated the staining (data not shown). Due to the diversity of cell types in brain tissue, there are many different cells in these primary brain cell cultures. Many of the cells have one or more branching dendritic or axon-like processes which contain fibers that stained intensely with anti-MAP2 and anti-tubulin antibody (Fig. 4). The fibers appear to contain bundles of microtubules because they splay out into thinner filaments at the termini of those processes that have contacted the substratum. Approximately 40% of the cells with epithelial or fibroblastic shape stained with the anti-MAP2 antibody, and there was no visible distinction between those that did and those that did not contain MAP2. In the cells that stained, dual fluorochrome labeling of tubulin and MAP2 showed identical fiber networks. This probably was not due to nonspecific association of the four different immunoglobulin preparations used on the cells because other cells on the same coverslip had typical microtubule arrays but no MAP2 staining (Fig. 4 *e* and *f*). After a week in culture, the brain cells were usually overgrown by fibroblastic or glial cells which did not contain MAP2.

CHO, 3T3, HeLa, and PtK1 cells did not stain with the anti-MAP2 antibody. To test whether MAP2 antigen was simply specific for mouse primary cells, mouse kidney explants were cultured and prepared for immunofluorescence in parallel with the brain cells. The kidney cells showed typical interphase fiber networks with anti-tubulin but no significant staining with anti-MAP2 antibody; furthermore, the mitotic spindles did not stain in these or any other cell examined (Fig. 4 *g* and *h*).

The neuroblastoma cell lines N18 and N2a had no detectable MAP2 antigen by immunofluorescence. Rat B104 neuroblastoma cells (21), which exhibit many biochemical characteristics associated with differentiated neurons, consistently stained with anti-MAP2 antibody. The staining intensity was intermediate between that of the primary brain cells and that of the controls (Fig. 5). Due to the round shape of these cells it was difficult to determine if the staining with anti-MAP2 and anti-tubulin was associated with cytoplasmic fibers. Anti-MAP2 antibody did not significantly stain the mitotic spindle in these cells. When the rat B104 cells were grown in the presence of 1 mM dibutyryl cyclic AMP (DBcAMP), they underwent morphological alterations that mimic neuronal differentiation (21). The dibutyryl cyclic AMP-treated cells stained more intensely with the anti-MAP2 antibody than did the untreated cells, indicating the appearance or perhaps the rearrangement of MAP2 in the developing neurons.

DISCUSSION

The present results demonstrate that hybridoma clone D-1D1.16.6 secretes antibody that is specific for the HMW neurotubule-associated protein MAP2. Within the limit of detection provided by the techniques used, there is no crossreactivity with other proteins from tissue and cell line homogenates. We have used indirect immunofluorescence rather than direct fluorochrome labeling of the anti-MAP2 antibody, even though the secondary antiserum may become the limiting factor in specificity, because the indirect method provides amplification of the image intensity. It is straightforward to control for nonspecific absorption of the secondary antiserum by the use of

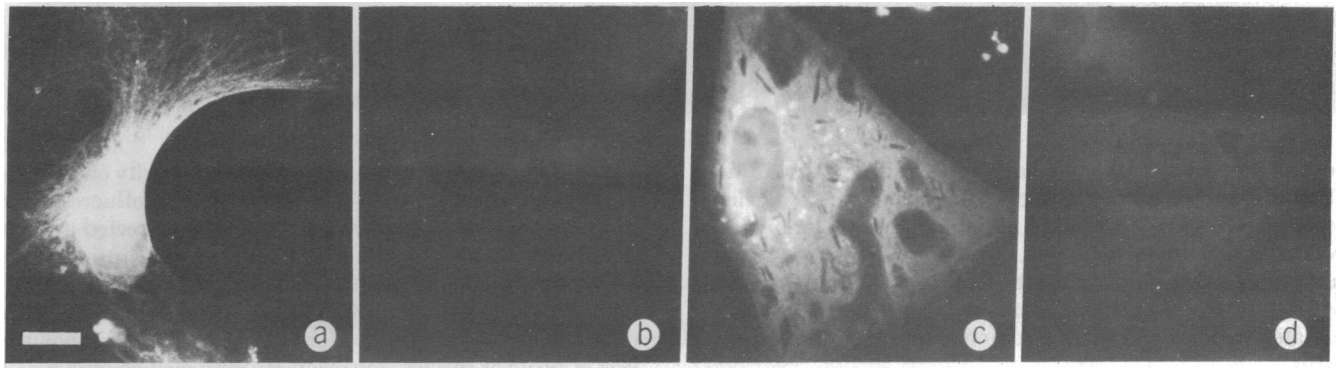

FIG. 3. Indirect immunofluorescence with anti-MAP2 antibody. (*a*) Mouse brain cell stained with anti-MAP2 antibody, showing array of interphase microtubules. (*b*) Mouse brain cell stained with control monoclonal antibody, showing nonspecific staining by fluorochrome-labeled rabbit anti-mouse preparation. (*c*) Mouse brain cell treated with 0.05 μg of the microtubule inhibitor nocodazole per ml for 30 min prior to staining with anti-MAP2 antibody. (*d*) 3T3 cells stained with anti-MAP2 antibody. (×730; scale bar is 10 μm.)

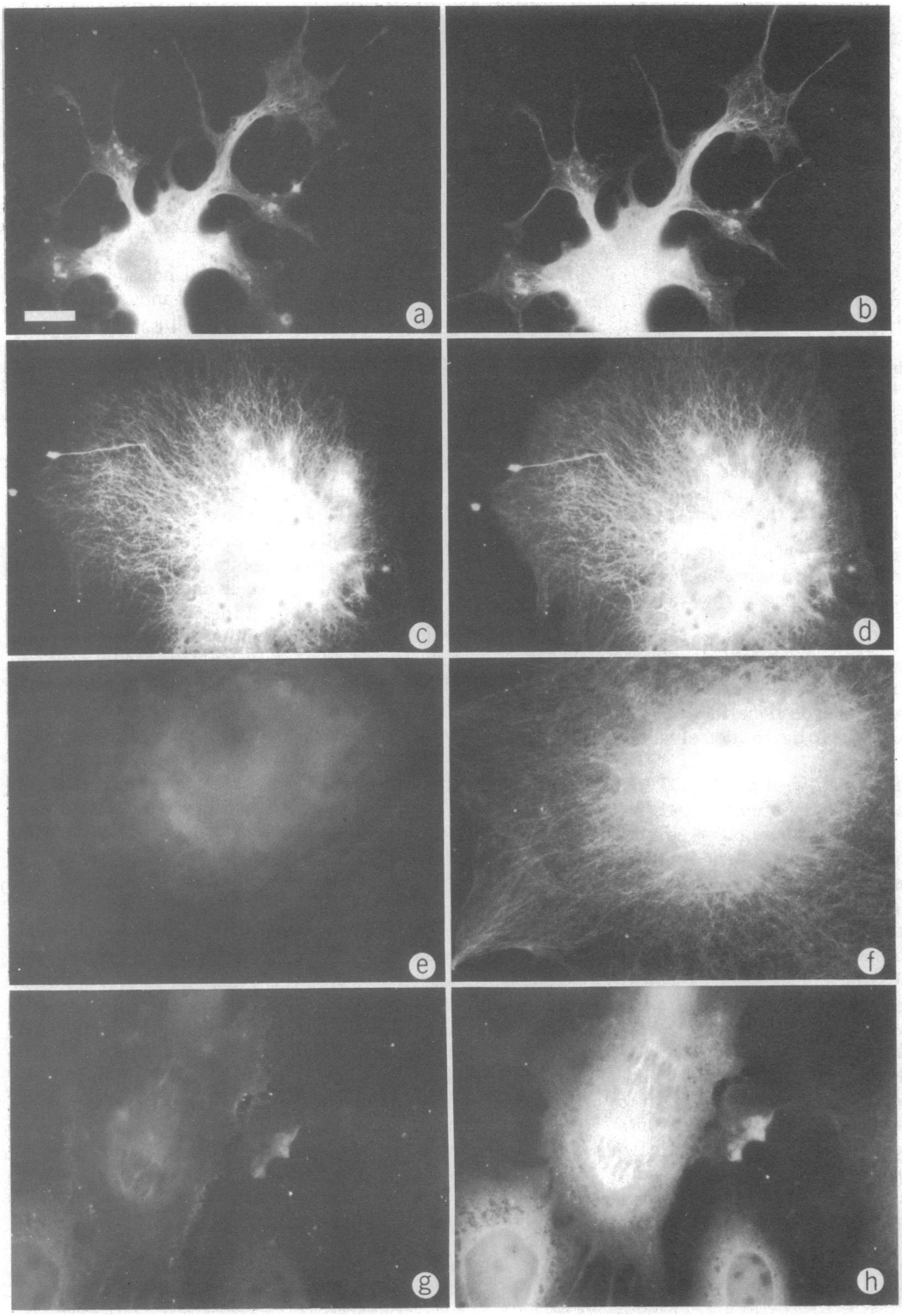

FIG. 4. Dual fluorochrome labeling of MAP2 and tubulin antigens. Primary cultures of BALB/c mouse cells were stained with anti-MAP2 (*a*, *c*, *e*, and *g*) and with anti-tubulin (*b*, *d*, *f*, and *h*). Many mouse brain cells showed identical patterns of MAP2 and tubulin staining (*a* and *b*; *c* and *d*); other cells in the same preparation did not stain with anti-MAP2 antibody (*e*) even though the tublin network appeared normal (*f*). Mouse kidney cells (*g* and *h*) and all of the mitotic spindles examined did not stain significantly with anti-MAP2 antibody. Micrographs *e* and *g* only show dim highlights when printed in the same fashion as the other micrographs. Thus, they have been printed at low contrast to document the lack of specific fibrilar MAP2 staining. (×730; bar is 10 μm.)

control monoclonal IgG. We have also avoided possible alteration of the monoclonal antibody specificity caused by fluorochrome labeling or affinity chromatography elution. The $NaDodSO_4$ antigen detection, radioimmunoassay, and immunofluorescence data indicate that the MAP2 antigen recognized by the D-1D1.16.6 antibody is present only in differentiated neurons and is not a component of the mitotic apparatus.

There have been reports that rabbit antisera raised against HMW stain interphase and mitotic spindle microtubules in a wide variety of cells (5, 6, 9). Although immunodiffusion and preabsorption experiments demonstrated the purity of the sera used in these studies, it is possible that the immunofluorescence images were contributed by immunoglobulin directed against trace components of the antigen which were distinct from HMW. Alternatively, the prevalent antibody in those preparations may have reacted with the larger HMW protein which might be highly conserved. More recent studies have shown a lack of tissue or species crossreactivity with well-characterized rabbit antisera to high molecular weight assembly MAPs (refs. 10 and 11; unpublished results). If these antisera are recognizing a subset of the antigenic determinants on the HMW mole-

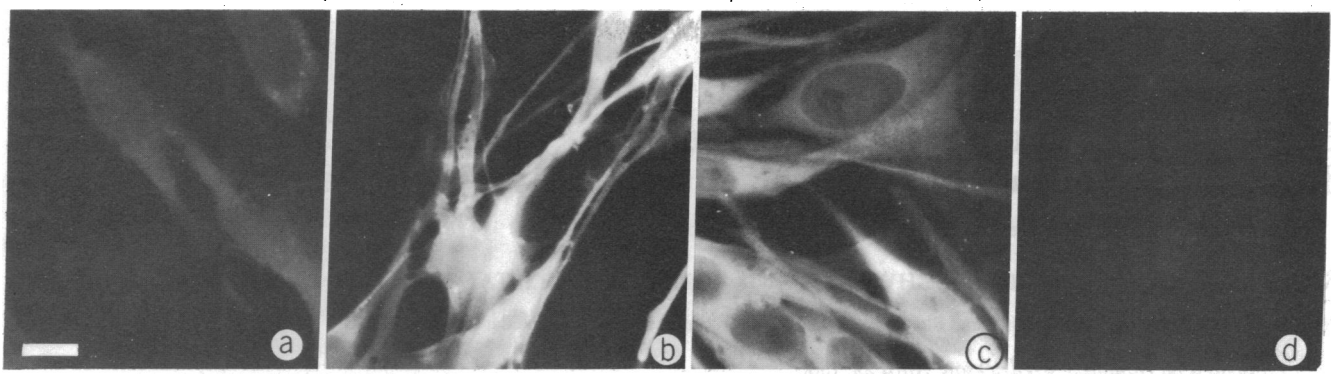

FIG. 5. Indirect immunofluorescence with rat B104 neuroblastoma cells. (*a*) B104 cells grown in normal culture medium and stained with anti-MAP2 antibody. (*b*, *c*, and *d*) B104 cells grown in the presence of 1.0 mM dibutyryl cyclic AMP and stained with anti-MAP2 (*b*), anti-tubulin (*c*), or nonspecific monoclonal antibody (*d*). (×730; bar is 10 μm.)

cules—and because the monoclonal anti-MAP2 antibody presumably recognizes a single determinant—then the reason for the discrepancy may be that the sera in the studies showing crossreactivity recognize other HMW determinants which are present on antigenically related MAPs in a wide variety of cells. Nonetheless, the present results continue to suggest that the MAP2 gene is expressed solely in differentiated neurons and demonstrate the ability of the monoclonal technique to resolve subtle differences in antigen structure.

MAP2 binds to microtubules with defined periodicity *in vitro* (13, 22). We have not observed any staining periodicity with anti-MAP2 antibody such as that seen with myosin antisera staining of cytoplasmic actin fibers (23). If MAP2 binding to microtubules is at saturation *in vivo* then the resulting periodicity may be below the resolution available from the immunofluorescence technique. It is hoped that electron microscopic immunocytochemical examination of brain explants will be able to detect periodicity if it is present.

The specific localization of MAP2 along microtubules in differentiated neurons raises the possibility that MAP2 is involved in some neuron-specific process. The MAP2 antigen appears associated with B104 cells after dibutyryl cyclic AMP treatment but there is no significant staining of the mitotic spindle of the dividing B104 cells with anti-MAP2 antibody. The association of MAP2 with specific subsets of cytoplasmic microtubules within a single cell type supports this hypothesis. MAP2 may function as an especially efficient microtubule polymerizing or stabilizing agent, promoting the formation of axonal and dendritic processes. This, in addition to the large tubulin content, may be a factor for the relative ease with which one can isolate microtubules from brain compared to other tissues and cell types. MAP2 may also be involved in axonal transport by crosslinking microtubules to other proteins. HMW has been shown to interact with actin in affinity chromatography (24) and viscometry experiments (25), suggesting that MAP2 could be involved in axonal transport. Because MAP2 forms arms when it decorates microtubules *in vitro* (13) it could also be involved in crosslinking microtubules to the membrane or other cellular components. Although it is impossible at present to define the role of MAP2 *in vivo*, the information available suggests that microtubule protein containing MAP2 may be a poor model system for the study of microtubule assembly and function in more ephemeral cytoplasmic systems such as the mitotic spindle.

Finally, the appearance or reorganization of MAP2 concomitant with dibutyryl cyclic AMP-induced differentiation of B104 neuroblastomas supports the hypothesis that MAP2 is important for the structure or function of neuronal cells. However, it is difficult to assess to what degree changes in the gene expression of a cultured tumor cell reflect normal developmental processes. The results do indicate that the MAP2 antigen may be a good marker for neuron development.

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