Widespread Distribution of a 210,000 mol wt Microtubule-associated Protein in Cells and Tissues of Primates

JEANNETTE CHLOË BULINSKI and GARY G. BORISY

Laboratory of Molecular Biology, University of Wisconsin, Madison, Wisconsin 53706. Dr. Bulinski's present address is the Department of Biochemistry, University of Washington, Seattle, Washington 98195.

ABSTRACT Antisera prepared against a 210,000 mol wt microtubule-associated protein (210k MAP) isolated from the human cell line, HeLa, were used to survey a variety of cells and tissues for the presence of immunologically related proteins. The antisera were employed to test extracts of the cells and tissues, using a sensitive indirect immunofluorescence technique applied to polyacrylamide gels. Cross-reactive material of 210,000 mol wt was found in 10 kinds of cells and tissues derived from humans and four lines of cells from monkeys. Indirect immunofluorescent staining was also carried out on fixed cells and showed that the cross-reactive material was localized to interphase and mitotic microtubules as assayed in nine human and seven monkey cell lines. No protein that cross-reacted with 210k MAP antisera was detected in cells and tissues derived from two rodents, an ungulate, a marsupial, or a chicken. Therefore, the 210k MAP isolated from HeLa cells is present in a wide variety of cells and tissues of humans and other primates but is antigenically distinct from MAPs present in lower organisms.

Using antisera prepared against HeLa microtubule-associated proteins (MAPs), we have previously demonstrated the association of 210,000 mol wt (210k) and 125k MAPs with HeLa microtubules polymerized in vitro and with colcemid-sensitive cytoplasmic fibers and mitotic spindles in vivo (1). Other experiments with these antisera have revealed that the 210k and 125k MAPs possess antigenic determinants not present on MAPs previously isolated from mammalian brain tissue. In this study, in an effort to determine whether or not the 210k MAP isolated from HeLa cells is unique to HeLa, we tested a variety of cells and tissues for the presence of 210k MAP using the same indirect immunofluorescent staining assays we employed for HeLa cells.

MATERIALS AND METHODS

Immunological Procedures

Preparation of antisera against HeLa 210k MAP and indirect immunofluorescent staining procedures for polyacrylamide gels and fixed cells have been described elsewhere (1).

Cell Culture

All cells were grown at 37°C in a humid atmosphere of 5% CO_2, 95% air. Medium was changed on cell monolayers every 2–3 d; cells were trypsinized (by exposure to a solution of 0.25% trypsin, 0.1% glucose, 0.01% EDTA in phosphate-buffered saline [5] lacking Ca^{++} and Mg^{++}, for 3 min) and subcultured every 3–7 d. Cells were plated on glass coverslips at least 24 h before being processed for immunofluorescence. Newborn human foreskin fibroblasts (strain 356), human diploid bladder endothelium (Ruba D), human tetrapolid bladder endothelium (Ruba T), human embryonic kidney, and mouse connective tissue (L-613 line) cells were the generous gift of Dr. Catherine Reznikoff and Dr. Robert De Mars (Department of Genetics, University of Wisconsin) and were grown in Ham's F-12 supplemented with 10% fetal calf serum. Human fetal glial cells and primary explants of fetal human brain, which contained neuronal and glial cells, were kindly prepared by Dr. Billie Lou Padgett and Dr. Duard Walker (Department of Medicine, University of Wisconsin) and were grown in Dulbecco's minimum essential medium containing 10% fetal calf serum. Human liver (Chang, catalog No. CCL13) and human neuroblastoma (IMR-32, catalog No. CCL127) were obtained from the American Type Culture Collection (Rockville, MD.), and were grown in Eagle's minimum essential medium containing Hanks' basal salt solution and 10% heat-inactivated fetal calf serum. A human myoblast clone, H240, was grown on gelatin-coated microscope slides by Dr. Stephen Hauschka (Department of Biochemistry, University of Washington, Seattle, Wash.), fixed by our normal methanol fixation (1), air dried, and mailed to us for staining with HeLa MAP antiserum. The African Green Monkey kidney cell line (CV-1) was obtained from Dr. Janet Mertz and was grown in Dulbecco's minimum essential medium supplemented with 0.1% glucose and 10% calf serum. The African Green Monkey kidney cell line (BSC-40) was a gift from Dr. Dennis Hruby (Biophysics Laboratory, University of Wisconsin). Abyssinian Colobus brain cells (line M916), Stumptail Macaque spleen cells (line M109), Owl Monkey kidney cells (line S618Q) and Ringtailed Lemur kidney cells (line M1273) were generously

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supplied by Dr. George Todaro (National Cancer Institute, Bethesda, Md.). The latter five cells were cultured in Dulbecco's minimum essential medium containing 20% heat-inactivated fetal calf serum. Because they attached poorly to normal glass coverslips, Owl Monkey and Ringtailed Lemur cells were grown on coverslips which were first coated with 1% poly-lysine, then rinsed in distilled water and sterilized. Mouse embryo fibroblasts (3T3) were donated by David Grunwald and Dr. Rex Risser (McArdle Laboratory, University of Wisconsin), and chick neuroretina and chick embryo fibroblasts were obtained from Jerry Grunwald and Dr. Jack Lilien (Department of Zoology, University of Wisconsin). Chinese hamster ovary (CHO) cells were obtained from Patricia Witt and rat kangaroo lung (PtK-1) cells were obtained from Paul Kronebusch, both of this laboratory. Mouse melanoma cells were obtained from Dr. Stuart MacPhail (Immunobiology Research Center, University of Wisconsin). The last six types of cells were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum.

Peripheral leukocytes from the Cotton-Topped Marmoset (line B958), which are permanently infected with Epstein-Barr virus, were not cultured in our laboratory, on account of the biohazard of the virus. The leukocytes, normally grown in suspension culture, were applied to poly-lysine-coated coverslips and allowed to attach overnight before fixation. We thank Willie Mark and Dr. Bill Sugden for preparing these cells for us.

Human sperm were obtained from Dr. Sander S. Shapiro (Infertility Clinic, Department of Gynecology, University of Wisconsin Hospital, Madison, Wis.). Sperm, $\sim 10^8$ cells/ml in seminal fluid, were applied to poly-lysine-coated coverslips and then rinsed in phosphate-buffered saline and processed as usual for immunofluorescence.

Pig brain tissue was obtained from Jones' Slaughterhouse, (Fort Atkinson, Wis.), and adult human brain tissue was obtained from the laboratory of Dr. J. R. Sheppard (Department of Genetics and Cell Biology, University of Minnesota, Minneapolis, Minn.).

Preparation of Cell Extracts

Cells were removed from monolayers with a rubber scraper, centrifuged at 1,000 *g* for 5 min, and washed three times with phosphate-buffered saline lacking Mg^{++} and Ca^{++}. Cells were resuspended in a ratio of 1:10 (wt/wt) in a sample buffer (6) containing 4% SDS, and were lysed by three 30-s pulses of sonication at setting 2 of a Heat Systems Sonifier (Branson Ultrasonics, Plainview, N. Y.). Cell lysates were boiled for 20 min, then applied to polyacrylamide slab gels.

RESULTS

The methods used to prepare the HeLa MAP immunogen and to characterize the antisera elicited in rabbits have been described in detail elsewhere (1). Briefly, purified microtubule protein was prepared by self-assembly from HeLa extracts, the 210k HeLa MAP was excised from SDS polyacrylamide gels, and the antisera were tested for specificity by indirect immunofluorescence binding assays. The 210k MAP antisera bound to a 210,000 mol wt species in HeLa cell extracts, bound to in vitro polymerized HeLa microtubules, and bound to a colcemid-sensitive fiber network in fixed HeLa cells. Thus, we concluded that the 210k HeLa protein was indeed associated with HeLa microtubules and distributed along their length in vivo.

To determine whether the 210k MAP identified in HeLa cells was specific to this cell line or of more general occurrence, we surveyed a variety of cells and tissues for material cross-reactive with the HeLa 210k MAP antisera. Two analytical methods, both using an indirect immunofluorescence technique, were employed. One method assayed the binding of HeLa MAP antisera to proteins separated by one-dimensional SDS polyacrylamide gel electrophoresis. This technique allowed us to identify cross-reactive material in extracts of cells and tissues and to determine its molecular weight. The second method assayed the binding of the antisera to fixed cells by conventional epifluorescence microscopy. This permitted us to determine the spatial distribution of any cross-reactive material present.

Extracts were prepared by sonication of cell or tissue suspensions directly into an electrophoresis buffer containing SDS (see Materials and Methods for details). By using this procedure, we hoped to avoid proteolysis of the 210k MAP and possible selective loss of cytoplasmic proteins. Each cell extract was electrophoresed in duplicate wells of a slab gel and additional wells contained samples of HeLa microtubule protein run as markers in parallel on each slab. After electrophoresis, one-half of each slab gel was stained with Coomassie Brilliant Blue and the other half was stained with 210k MAP antiserum followed by fluorescently conjugated second antibody.

Fig. 1 shows the electrophoretic profiles obtained for eleven cell and tissue extracts derived from humans or African Green Monkeys. The HeLa microtubule protein standard is shown for the first extract, but for clarity has been omitted for the others. However, the black line denotes the position of the HeLa 210k MAP as run in each parallel marker lane. In each of the extracts shown in Fig. 1, a protein of ~210,000 mol wt that cross-reacted with the 210k MAP antisera was visualized. Note that in many cases (e.g., human foreskin, liver, fetal glial, adult brain, diploid bladder, and fetal kidney), no strong Coomassie-stained band is evident in the immediate region of the 210k band. In other cases (e.g., tetraploid bladder, neuroblastoma, fetal brain, and African Green Monkey kidney) Coomassie-stained bands are evident nearby, but close inspection indicates that these bands do not align precisely with the fluorescent band. Hence, the cross-reactive material identified in these extracts most likely represents a component present at low proportion relative to total protein.

We previously demonstrated that <10 ng of 210k HeLa MAP could be detected on gels stained by this immunofluorescence technique. We have not attempted to quantitate exactly the amount of cross-reactive material in the various cell types, but, by comparison with the fluorescence intensity of the parallel HeLa MAP marker in each run, a rough estimate is possible. Assuming antigenic identity, we estimate that the MAP content of the 11 cell and tissue types assayed ranges between 0.03 and 0.07% of soluble cell protein, which compares to ~0.04% for HeLa. The two cell strains that appeared to contain the greatest proportions of MAPs were Chang liver (panels *g* and *h*), and fetal glial (panels *m* and *n*). Although 210k MAP was detected in all of the extracts shown in Fig. 1, it was not detected in extracts of CHO, mouse (3T3, melanoma, and L-613), or rat kangaroo (PtK-1) cells or in pig brain tissue (data not shown). The same immunofluorescent staining procedure was used for these nonprimate cell extracts as for the primate extracts shown in Fig. 1. Because we applied as much as 150 μg protein to the gels, the failure to detect a fluorescent band implies that antigenically identical 210k MAP, if present, constituted <0.007% of the soluble cell protein of these nonprimate cells and tissues.

We also examined various kind of fixed cells for the presence of 210k MAP associated with cytoplasmic microtubules and mitotic spindles. Fig. 2 displays some examples of indirect immunofluoresence patterns of human and monkey cells. The patterns shown indicate that cross-reactive material was associated with a fiber network in interphase and mitotic cells. In interphase, the network focused to a region near the nucleus and extended toward the margin of the cells. The pattern in dividing cells corresponded to the distribution of fibers in the mitotic spindle and midbody. A cytoplasmic network was not detected in cells stained with preimmune serum or in cells pretreated with Colcemid (data not shown). Parallel cultures of cells stained with antibody to tubulin showed the same fiber network as with the 210k MAP antisera. Thus, we conclude

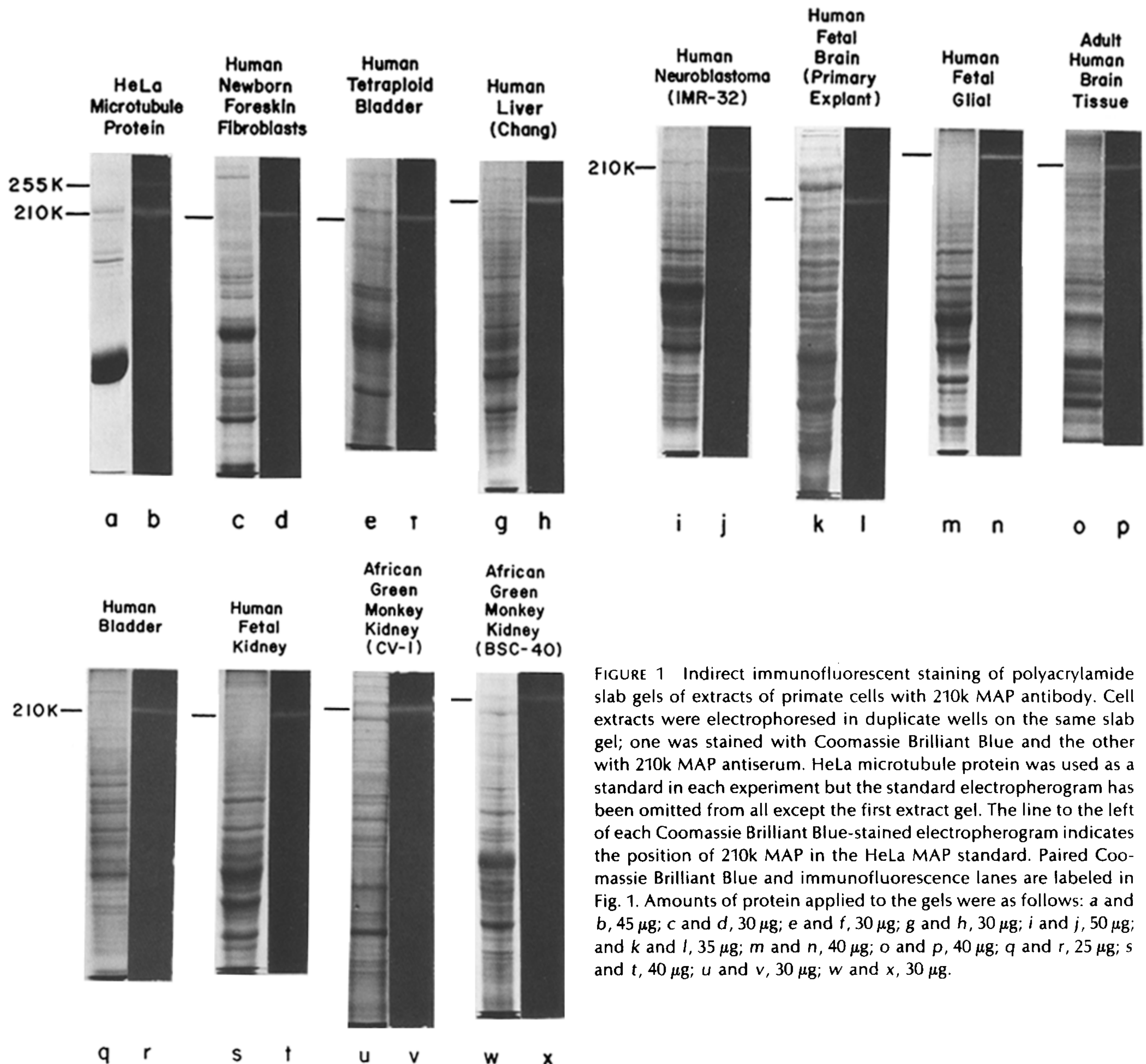

FIGURE 1 Indirect immunofluorescent staining of polyacrylamide slab gels of extracts of primate cells with 210k MAP antibody. Cell extracts were electrophoresed in duplicate wells on the same slab gel; one was stained with Coomassie Brilliant Blue and the other with 210k MAP antiserum. HeLa microtubule protein was used as a standard in each experiment but the standard electropherogram has been omitted from all except the first extract gel. The line to the left of each Coomassie Brilliant Blue-stained electropherogram indicates the position of 210k MAP in the HeLa MAP standard. Paired Coomassie Brilliant Blue and immunofluorescence lanes are labeled in Fig. 1. Amounts of protein applied to the gels were as follows: *a* and *b*, 45 μg; *c* and *d*, 30 μg; *e* and *f*, 30 μg; *g* and *h*, 30 μg; *i* and *j*, 50 μg; and *k* and *l*, 35 μg; *m* and *n*, 40 μg; *o* and *p*, 40 μg; *q* and *r*, 25 μg; *s* and *t*, 40 μg; *u* and *v*, 30 μg; *w* and *x*, 30 μg.

that the 210k MAP is associated with microtubules in human and monkey cells.

Panels *a* and *b* in Fig. 2 show two examples of human foreskin fibroblasts. The fibers focus on an area near the nucleus, the microtubule-organizing center (MTOC), and extend to the edges of these extremely flat cells. Panel *b* resembles microtubule patterns observed in locomoting cells, while panel *a* is more typical of sessile cells. The lovely metaphase and anaphase examples (panel *c*) are common in this human fetal kidney strain and in the other four primate kidney lines, in which many cells remain somewhat flat during mitosis.

The colony of human bladder cells shown in panel *d* demonstrates that fibrous staining patterns are not rare. In fact, the fibrous patterns illustrated in Fig. 2 are representative of 16 primate cell lines examined (see Table I). Panel *e* shows a tetraploid bladder cell. These cells resemble the diploid bladder cells, and in this example the MTOC can be seen over the nucleus. Panel *f* contains a human fetal neuronal cell. The branched neurites stain brightly, while the cell body contains fewer, less organized microtubules.

Panels *g* and *h* contain Chang liver cells in two stages of cell division. In the metaphase spindle (panel *g*) astral rays are faintly seen. The midbody in panel *h* is an elaborate structure common in Chang cells, in which the midbody persists far into the G_1 period of the next cell cycle. The MTOCs of the two daughter cells are visible with the cytoplasmic microtubules already reforming. Panels *i* and *j* show African Green Monkey kidney cells, a colony of the CV-1 line (panel *i*) and a single BSC-40 cell (panel *j*). MTOCs can be seen and individual fibers can be traced in some of these cells. Panel *k* shows a typical anaphase figure in a BSC-40 cell. The region in the center of the spindle that shows slightly fainter staining may be a precursor of the faintly staining center of the midbody. In the two daughters, cytoplasmic microtubules are already forming.

A Colobus brain cell can be seen in panel *l*. This line, isolated from brain tissue, appears from its extremely flat morphology and from the large size of its cells, to be glial in

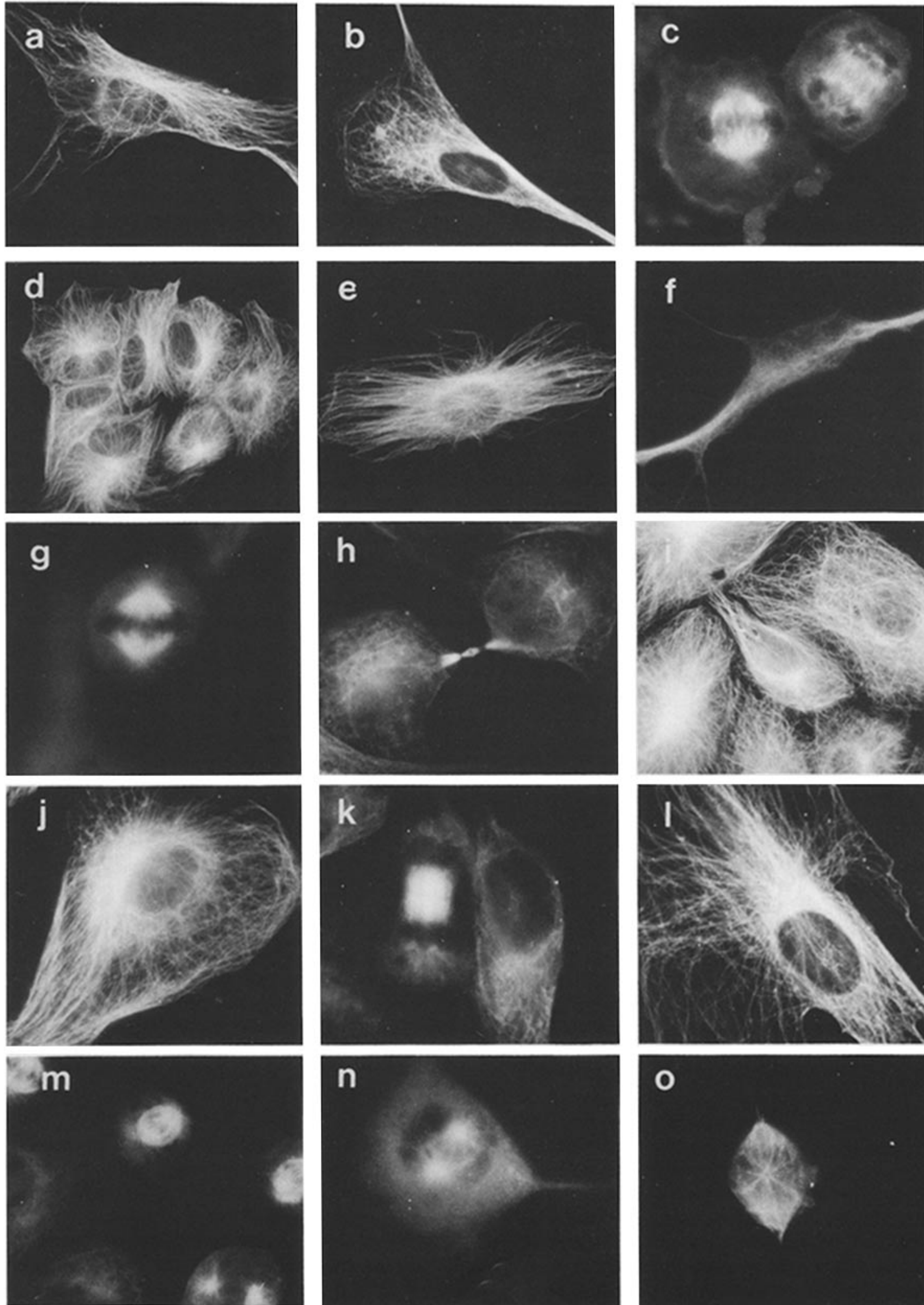

FIGURE 2 Indirect immunofluorescent staining of primate cells with 210k MAP antibody. (*a*) Interphase human foreskin fibroblast. × 430. (*b*) Interphase human foreskin fibroblast. × 370. (*c*) Mitotic human fetal kidney. × 675. (*d*) Colony of interphase human bladder. × 260. (*e*) Interphase human tetraploid bladder. × 460. (*f*) Human fetal neuronal. × 770. (*g*) Human liver (Chang) metaphase. × 670. (*h*) Human liver (Chang) showing midbody. × 670. (*i*) Colony of interphase African Green Monkey kidney (CV-1). × 460. (*j*) Interphase of African Green Monkey kidney (BSC-40). × 530. (*k*) Anaphase African Green Monkey (BSC-40). × 700. (*l*) Interphase Colobus brain. × 580. (*m*) Stumptail Macaque spleen showing mitotic figures. × 350. (*n*) Owl Monkey prometaphase. × 920. (*o*) Interphase Cotton-Topped Marmoset. × 1,160.

origin. Panel *m* shows mitotic spindles of Stumptail Macaque spleen cells. A prophase figure is in the lower right-hand corner and there are three metaphase figures as well. Another prophase spindle is shown in panel *n*, an Owl Monkey kidney cell. This cell is beginning mitosis but still has an extremely flat morphology, like many kidney cells. The last panel, *o*, shows a peripheral leukocyte from a Cotton-Topped Marmoset. Note that the probable MTOC at the center of an astral-like complement of cytoplasmic microtubules is a region of reduced staining.

The only primate cell examined that failed to stain was human sperm (not shown). The 210k MAP is apparently not present in the microtubules of the sperm tail flagellum.

Cells derived from organisms other than primates were also tested for the presence of 210k MAP. No staining was observed in nonprimate cells. To illustrate this negative result in a hopefully convincing manner, we prepared mixed cultures of human and nonprimate cells. Fig. 3 shows two such mixtures, HeLa and rat kangaroo (PtK-1) cells and HeLa and mouse (L-613) cells. Because we previously tested each of the types of cells shown in Fig. 3 in single populations, we could identify the human and nonprimate cells in mixed cultures by their staining behavior, as well as sometimes by their characteristic morphologies. Both pairs of phase and immunofluorescence images are meant to demonstrate the staining of the human but not the nonprimate cells in the field.

Table I summarizes the distribution of the 210k MAP in cells and tissues derived from various animals. 19 cell types were examined by indirect immunofluorescence of gels, 24 cell types were examined by indirect immunofluorescence of cells, and 16 cell types were examined by both procedures. The 210k MAP was present in the cytoplasmic microtubules, mitotic spindles, and midbodies of epithelial and fibroblastic human cells, in the cytoplasmic microtubules of human myoblast cells, and in the neurite extensions of primary fetal neuronal cells and human neuroblastoma cells. We have not observed cross-reactive material in the flagellar microtubules of human sperm tails. Therefore, 210k MAP antigenically similar to that isolated from HeLa is present in at least the somatic cells and tissues of primates.

DISCUSSION

In this paper, we have shown that the 210k MAP which we have isolated from HeLa cells is associated with the microtubules of somatic human and monkey cells. The 210k MAP is apparently associated with all of the microtubules of the cytoplasmic microtubule complex in interphase cells and the spindle and midbody in dividing cells. Therefore, the association of the MAP with microtubules is not restricted to a particular stage of the cell cycle. Although the cells (HeLa) from which the 210k MAP was originally isolated were derived from a tumor (cervical carcinoma), an antigenically similar MAP was detected both in cells of tumor and nontumor origin. All of the primate cells with the exception of neuroblastoma were originally derived from nonmalignant tissues. Of these, all of the monkey cells and the Chang liver cells are established cell lines. However, the human adult brain tissue represents material from cells that were never grown in culture and the human embryonic brain cells were obtained from a primary explant. The foreskin fibroblasts, kidney, glial, bladder, and myoblast cells were obtained at low passages and all retained normal morphology and karyotype. Therefore, the 210k MAP is not indicative of a transformed state or of cells in culture, but rather is a component of apparently normal cells.

The presence of the MAP was also not restricted to a particular type of tissue. The MAP was detected in cells and tissues of connective tissue (human foreskin fibroblasts, Cotton-Topped Marmoset leukocytes), nervous tissue (human adult brain, human embryonic brain, Abyssinian Colobus brain, human fetal glial and human neuroblastoma), muscle tissue (human myoblasts and myotubes), and in epithelial tissue (eight cell types in addition to HeLa; see Table I). These tissues represent all of the four principal types of body tissue. The only primate cell type tested that was negative was human sperm. Other stages in the development of the gametes have not been tested and it is not possible to conclude that the 210k MAP is absent from the germ line. Nevertheless, it is clear that

TABLE I

Detection of 210k MAP in Cells and Tissues

Species of origin	Cell type or tissue of origin	Immunofluorescence assay of extracts on gels	Immunofluorescence assay of fixed cells
Human	Cervical carcinoma (HeLa)	+	+
Human	Liver (Chang)	+	+
Human	Newborn foreskin fibroblast	+	+
Human	Diploid bladder endothelium (Ruba D)	+	+
Human	Tetraploid bladder endothelium (Ruba T)	+	+
Human	Fetal kidney	+	+
Human	Fetal glial	+	+
Human	Embryonic brain (Primary explant)	+	+
Human	Adult brain tissue	+	NA
Human	Neuroblastoma (IMR-32)	+	NA
Human	Myoblast (clone H240)	NA	+
Human	Sperm	NA	−
African Green Monkey	Kidney (CV-1)	+	+
African Green Monkey	Kidney (BSC-40)	+	+
Abyssinian Colobus	Brain (M916)	NA	+
Stumptail Macaque	Spleen (M109)	+	+
Cotton-Topped Marmoset	Peripheral leukocyte (B958)	NA	+
Owl Monkey	Kidney (S618Q)	NA	+
Ringtailed Lemur	Kidney (M1273)	+	+
Mouse	Connective tissue fibroblast (L-613)	−	−
Mouse	Embryo Fibroblast (3T3)	−	−
Mouse	Melanoma	NA	−
Chinese hamster	Ovary (CHO)	−	−
Pig	Brain tissue	−	NA
Rat kangaroo	Lung (PtK-1)	−	−
Chicken	Embryo fibroblast	NA	−
Chicken	Neuroretina	NA	−

+, Detected; −, not detected; NA, not assayed.

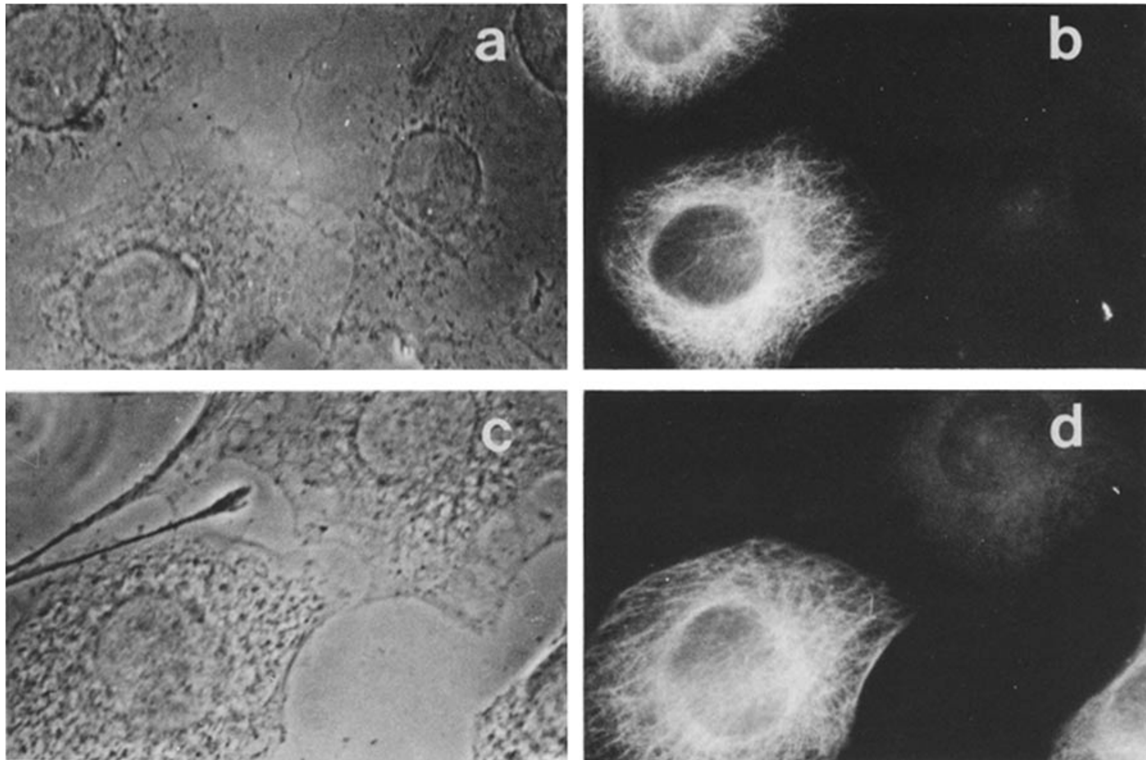

FIGURE 3 Phase and immunofluorescence pairs of human and nonprimate cells. Mixed cultures (~1:1 ratio) were seeded onto coverslips, allowed to spread for 1–2 d, then immunofluorescent stained. (*a* and *b*) Human cervical carcinoma and rat kangaroo cells. × 760. (*c* and *d*) Human cervical carcinoma and mouse connective tissue cells. × 900.

the MAP is widespread among primate somatic tissue.

In contrast to the primates, no cells or tissue derived from nonprimate sources scored positive. However, the absence of cross-reactive molecules should not be interpreted to signify that similar MAPs do not exist in nonprimate cells. Possibly, related molecules may be present that lack the determinants recognized by our antisera. Because the antisera were elicited in rabbits against a human antigen, the antibody molecules expressed may have been those directed against determinants not shared by humans and rabbits.

What is the relationship of the 210k MAP to the previously described high molecular weight (HMW) MAP isolated from vertebrate brain tissue? While it remains possible that the two molecules are related, there is no positive evidence for this hypothesis and the available evidence has only revealed differences. The HeLa 210k MAP antisera fail to react with HMW, and pig brain HMW antisera fail to react with 210k MAP (1). While the negative result for reaction of the HeLa antisera with the pig brain MAP can be accounted for on the basis of the primate specificity of the HeLa antisera, the failure of the reciprocal reaction cannot be similarly explained. The antisera raised against pig brain MAP have been shown to cross-react with brain MAPs from a variety of vertebrate species, including humans (7).

Previous work[1] has shown differences in molecular weight, sedimentation coefficient, and association properties of the HMW and 210k MAPs. When taken with the present study that has shown immunological differences, it seems probable that the two MAPs are not closely related molecules.

Previous immunocytochemical studies on the distribution of brain MAPs have indicated that these molecules are present on the cytoplasmic and spindle microtubules of a variety of cultured cells derived from pig (10), rat, and mouse (3, 4, 8, 9). However, in contrast, two recent studies have suggested that the HMW MAP is not an abundant component of a number of cultured cell lines of nonneuronal origin (reference 7 and footnote 2). The explanation for this difference is not clear; the two recent studies were performed using pure HMW as the immunogen, wheres earlier studies may have used preparations that contained other proteins as well. Possibly antibodies obtained were not directed solely against HMW. Further studies will be required to resolve the apparent discrepancy. The salient point for this paper, though, is that, unlike the 210k MAP, which is of widespread distribution in the cells and tissues of primates, the occurrence of HMW may be restricted to certain specialized cells (e.g., neuronal).

In at least one type of cell, human fetal neuronal, both HMW and 210k MAP have been shown to coexist by immunofluorescence.[3] Therefore, the distributions of HMW and 210k MAP are not mutually exclusive. A recent report (2) provides evidence that both the brain tau and HMW MAPs are present in a single non-neuronal cell type, mouse SV 3T3. Whether tau and 210k MAP coexist in a single cell type or indeed whether all three MAPs may be present together remains to be determined.

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Note Added in Proof: A recent paper by Sloboda and Dickerson (1980, *J. Cell Biol.* 87:170–179) has appeared providing evidence for the localization of MAP2 in the marginal band of nucleated erythrocytes.

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