Heterogeneity among Microtubules of the Cytoplasmic Microtubule Complex Detected by a Monoclonal Antibody to Alpha Tubulin

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ABSTRACT Three monoclonal antibodies specific for tubulin were tested by indirect immunofluorescence for their ability to stain cytoplasmic microtubules of mouse and human fibroblastic cells. We used double label immunofluorescence to compare the staining patterns of these antibodies with the total microtubule complex in the same cells that were stained with a polyclonal rabbit antitubulin reagent. Two of the monoclonal antitubulin antibodies bound to all of the cytoplasmic microtubules but Ab 1-6.1 bound only a subset of cytoplasmic microtubules within individual fixed cells. Differential staining patterns were observed under various fixation conditions and staining protocols, in detergent-extracted cytoskeletons as well as in whole fixed cells. At least one physiologically defined subset of cytoplasmic microtubules, those remaining in cells pretreated for 1 h with 5 μM colcemid, appeared to consist entirely of Ab 1-6.1 positive microtubules. The same was not true of the microtubules that remained in either cold-treated cells or in cells that had been exposed to hypotonic medium. The demonstration of antigenic differences among microtubules within single fixed cells and the apparent correlation of this antigenic difference with at least one "physiologically" defined subset suggests that mechanisms exist for the differential assembly or postassembly modification of individual microtubules in vivo, which may endow them with different physical or functional properties.

In addition to the established involvement of microtubules in mitosis and in flagellar and cilliary movement, cytoplasmic microtubules appear to play important roles in secretion, slow axonal flow, organelle translocation, supporting cellular asymmetries, and regulating cellular growth cycles (9, 10, 12). The involvement of microtubules in such diverse processes has suggested the hypothesis that particular tubulin variants might be preferentially polymerized into organelle-specific microtubules (14). Support for this "multi-tubulin hypothesis" has come from observations showing special tubulin synthesis for flagellar microtubules (17), the polymerization of particular isotubulins into cilia (6), the age-dependent distribution of tubulin isoelectric variants at different stages of neural development (7), and the discrete distribution of tubulin variants within certain neurites (5, 16).

Immunofluorescence microscopy using antibodies specific for the microtubule protein, tubulin, serves to define the spatial arrangement of microtubules in cells that assume a flattened shape in culture (4). These immunofluorescence procedures can detect even single microtubules coated with the antibody reagents (19). Interphase fibroblastic cells have been shown to contain a complex array of cytoplasmic microtubules, many of which appear to radiate from a pericentriolar region, referred to as the microtubule organizing center (4). Because these earlier studies were dependent upon polyclonal antibodies to tubulin, they were not designed to detect antigenic or functional differences among the constituent microtubules of this complex. Characterization of any such differences between cytoplasmic microtubules should aid our eventual understanding of how this complex array of microtubules is involved in various cellular functions.

To establish more highly specific tools with which to probe the structure and function of microtubules, one of us (D. J. Asai) recently developed a library of monoclonal anti-

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bodies to tubulin, using sea urchin sperm axonemes as the source for the antigen (1). We have shown that some monoclonal anti-alpha-tubulin antibodies differ among themselves in relative reactivities for denatured alpha-tubulins from different tissue sources, both between species and within a single species (2). One of these anti-alpha-tubulin antibodies, Ab 1-6.1, bound to microtubules in chick fibroblasts, but not to microtubules in PtK_2 cells, as shown by immunofluorescence. A high degree of selectivity was exhibited between species variants of microtubules. Pursuing this initial observation, we now report that Ab 1-6.1 binds to only a subset of the microtubules within single fixed mouse or human cells, indicating that the cytoplasmic microtubule complex contains antigenically different types of microtubules and microtubule segments. A preliminary report of some of these results has been presented (23).

MATERIALS AND METHODS

Materials: Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise. The affinity-purified polyclonal rabbit antitubulin reagent (13) was provided by Dr. Gerald M. Fuller (The University of Texas Medical Branch at Galveston).

Monoclonal Antibody Production: The production of monoclonal antibodies to tubulin has been described previously (1). Spleen cells for hybridoma production were taken from a mouse previously immunized over a 40-d schedule with a 0.6 M KCl extract from sea urchin (*Strongylocentrotus purpuratus*) spermatozoan axonemes that contained tubulin, dyneins, and several other proteins. Hybridomas producing antitubulin antibodies were cloned by limiting dilution and then expanded by ascitic growth in mice that had been primed with pristane (Aldrich Chemical Co., Milwaukee, WI). Ascites sera were collected and stored at −80°C.

Characteristics of Monoclonal and Polyclonal Antitubulin Reagents: Four antibodies, all specific for tubulin, were used in this study. The first monoclonal antitubulin reagent, Ab 1-6.1, has previously been shown to bind to only the alpha chain of tubulin by immunoautoradiography of western blots of SDS polyacrylamide gels (2). This antibody, Ab 1-6.1, has been characterized as an immunoglobulin of the class IgG1 according to the following criteria: (*a*) the antibody did not bind to protein A directly; (*b*) the typical Ab 1-6.1 immunofluorescence staining pattern was obtained when cytoskeletons were stained by a "triple-sandwich" combination of Ab 1-6.1, then unlabeled rabbit anti-mouse IgG1, followed by rhodamine-labeled goat anti-rabbit IgG antibodies; and (*c*) no staining of cytoplasmic microtubules resulted from triple-sandwich staining that utilizes either rabbit anti-mouse IgA or rabbit anti-mouse IgM as the second antibody reagent. The second monoclonal antitubulin reagent, Ab 1-2.3, also binds only to the alpha chain of tubulin (2). Monoclonal Ab 1-6.1 and 1-2.3 differ in their preferences for binding to alpha tubulins isolated from various sources (2), but these differences were not apparent on immunoautoradiographs of blots of tubulin subclasses from bovine brain tubulin separated by isoelectric focusing procedures or by two dimensional gel electrophoresis (unpublished results). The third monoclonal antitubulin, Ab 1-1.1, was specific for tubulin and avidly bound to all microtubules examined. The subunit specificity, however, has not yet been determined. The polyclonal rabbit antitubulin reagent consists of polyclonal rabbit antitubulin antibodies, purified from rabbit antiserum by affinity chromatography on tubulin-Sepharose (13). This polyclonal antitubulin reagent has previously been extensively used to stain the entire cytoplasmic microtubule complex in various cells (4, 13).

Cell Culture: Primary cultures of fibroblastic cells were prepared from the body walls of 11-d mouse embryos or from neonatal human foreskin tissues as described (8). The cells were maintained and passaged in Dulbecco Voght-modified Eagle's medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% calf serum (Irvine Scientific Co., Irvine, CA), penicillin and streptomycin. Secondary cultures were subcultured onto No. 1 glass coverslips in individual 35-mm culture dishes (6×10^4 cells/dish) one day prior to staining.

Extraction of Cytoskeletons: Extraction steps were carried out at 32°C except as noted. Coverslips with cells were rinsed briefly with PBS and then in warm microtubule-stabilizing buffer: 0.1 M PIPES, pH 6.9, 1 mM EGTA, and 4% polyethylene glycol (PEG-6000). The cells were then extracted for 5 min with 0.5% Triton X-100 in microtubule-stabilizing buffer containing aprotinin (0.1 trypsin inhibitor U/ml). The resulting cytoskeletons were routinely fixed for 10 min in a solution of 4% formaldehyde (freshly prepared from paraformaldehyde) in the microtubule-stabilizing buffer, then rinsed in PBS, immersed in −20°C acetone for 15 min, and finally rehydrated in PBS.

Indirect Immunofluorescence Staining: Antibody dilutions were made in 15 mM HEPES-buffered Dulbecco Voght-modified Eagle's medium containing 0.5% BSA, pH 7.4. The primary antibody reagents were the monoclonal antitubulin ascites sera, diluted 10- to 50-fold, and a well-characterized affinity-purified polyclonal rabbit antitubulin reagent (14), diluted to 10 μg/ml. Coverslips containing cytoskeletons or permeabilized whole cells were inverted over a drop of the diluted primary antibodies and incubated for 1 h at 37°C, and then rinsed extensively with PBS. Unless otherwise noted, when double immunofluorescence was performed, both primary antibodies were added simultaneously. For double fluorescence staining (except as noted) the coverslips were then incubated for 1 h at 37°C with the following combination of secondary antibodies: (*a*) fluorescein-labeled, affinity-purified, goat anti-rabbit IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD; lot CC09, 10 μg/ml) and (*b*) rhodamine-labeled goat anti-mouse IgA, IgG, and IgM (Cappel Laboratories, Cochranville, PA; lot 17019, diluted 50- or 100-fold). After rinsing with PBS containing 0.5% Tween 20, and then PBS, the coverslips were mounted in 10% glycerol in a borate buffered saline: 0.145 M NaCl, 50 mM H_3BO_3, 25 mM $Na_2B_4O_7$, adjusted to pH 9.0 with NaOH.

Fluorescence photomicrographs were made with a Leitz Orthoplan microscope equipped with a 63/1.30 Fluoresczenz oil immersion objective, epi-illumination using the filter combination cubes $N_{2.1}$ and I_2, and an Orthomat 35 mm camera. Kodak Tri-X film was developed in Ethol Blue (Ethol Chemicals, Chicago, IL) at an effective film speed of ASA 2000 (daylight).

RESULTS AND DISCUSSION

Immunofluorescent Staining of Cytoplasmic Microtubules

Earlier immunofluorescence studies have shown that a monoclonal antibody to alpha tubulin (Ab 1-6.1) specifically stains microtubules in chick embryo cells but does not stain microtubules in PtK_2 cells (2). Thus, this antibody can recognize species-specific antigenic differences between cytoplasmic microtubules. In an attempt to determine whether antigenically different microtubules might normally be found within the same cell, we examined the ability of Ab 1-6.1 and

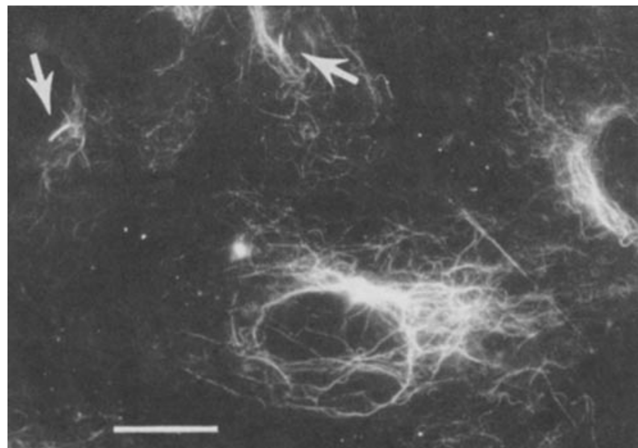

FIGURE 1 Indirect immunofluorescence staining of cytoplasmic microtubules using monoclonal antitubulin Ab 1-6.1. Coverslip cultures of ME cells were extracted with 0.5% Triton X-100 in microtubule stabilizing buffer, fixed in 4% formaldehyde, and extracted with cold acetone as described in Materials and Methods. The resulting cytoskeletons were then incubated with diluted ascites serum containing Ab 1-6.1, a monoclonal antibody to alpha tubulin. The fluorescent second antibody reagent was rhodamine-labeled goat anti-mouse immunoglobulins. This monoclonal antibody stains the primary cilium (arrows) and some cytoplasmic microtubules, but apparently does not stain the entire cytoplasmic microtubule complex. Bar, 20 μm. × 700.

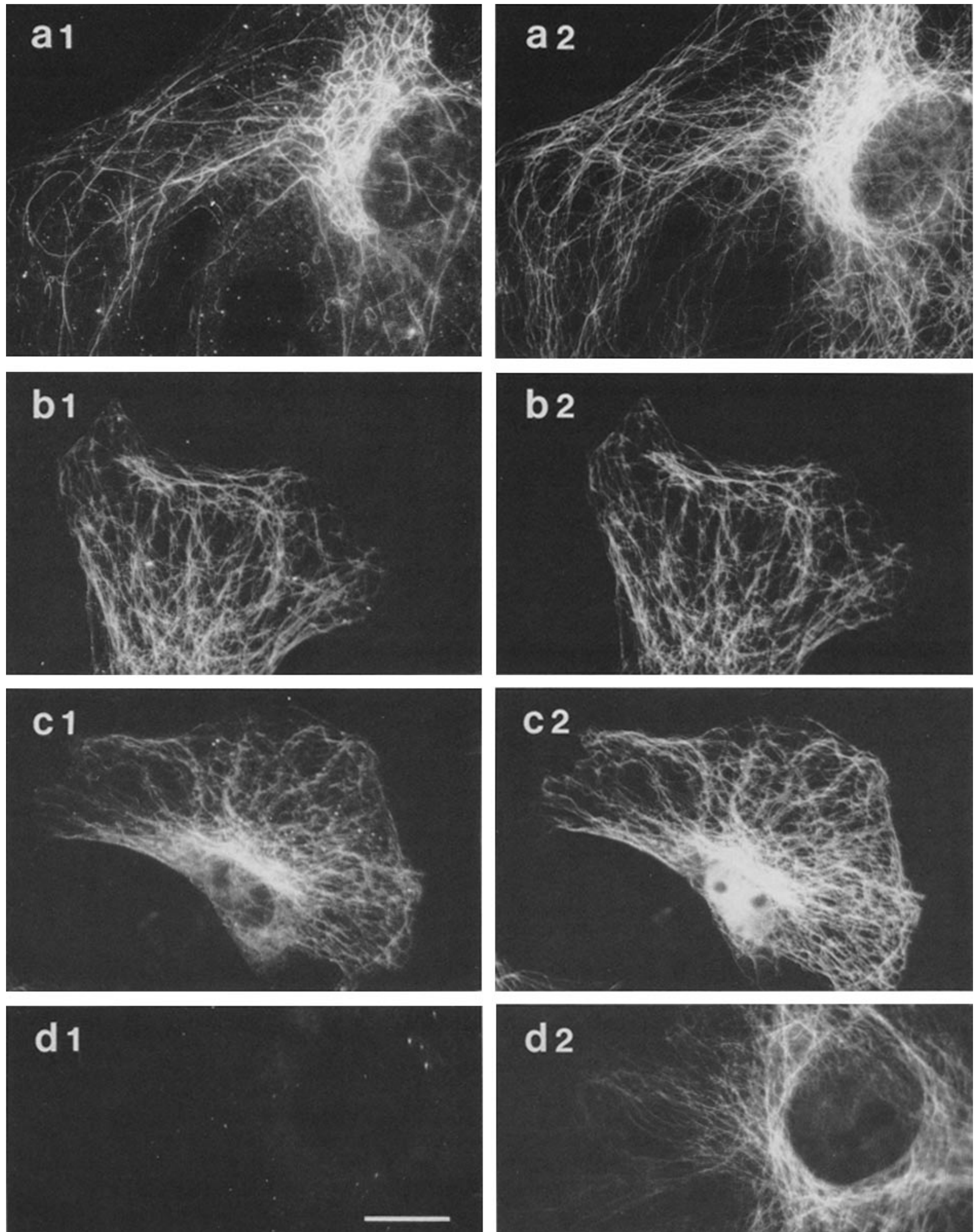

FIGURE 2 Immunofluorescent staining of some or all cytoplasmic microtubules in ME cells by three different monoclonal antitubulin antibodies. Detergent-extracted ME cell cytoskeletons prepared as in Fig. 1 were stained by double-label indirect immunofluorescence using rabbit polyclonal antitubulin antibodies in combination with one of the three different murine monoclonal antitubulin antibodies, followed by a combination of rhodamine-labeled goat anti-mouse immunoglobulins and fluorescein-labeled goat anti-rabbit IgG (see Materials and Methods). For each pair of fluorescence photomicrographs, the murine monoclonal antitubulin antibody staining pattern is shown in the first panel and the corresponding image of the entire cytoplasmic microtubule complex of that cell, as stained with polyclonal rabbit antitubulin antibodies, is shown in the second panel. *a1*, Monoclonal antibutulin Ab 1-6.1; *a2*, polyclonal antitubulin; *b1*, monoclonal antitubulin Ab 1-1.1; *b2*, polyclonal antitubulin; *c1*, monoclonal antitubulin Ab 1-2.3; *c2*, polyclonal antitubulin; and *d1*, no murine monoclonal antitubulin antibodies; *d2*, polyclonal antitubulin. By these comparisons, Ab 1-6.1 is shown to stain only a subset of the cytoplasmic microtubules, whereas the other two monoclonal antitubulin antibodies appear to stain all of the microtubule complex. Bar, 20 μm. × 700.

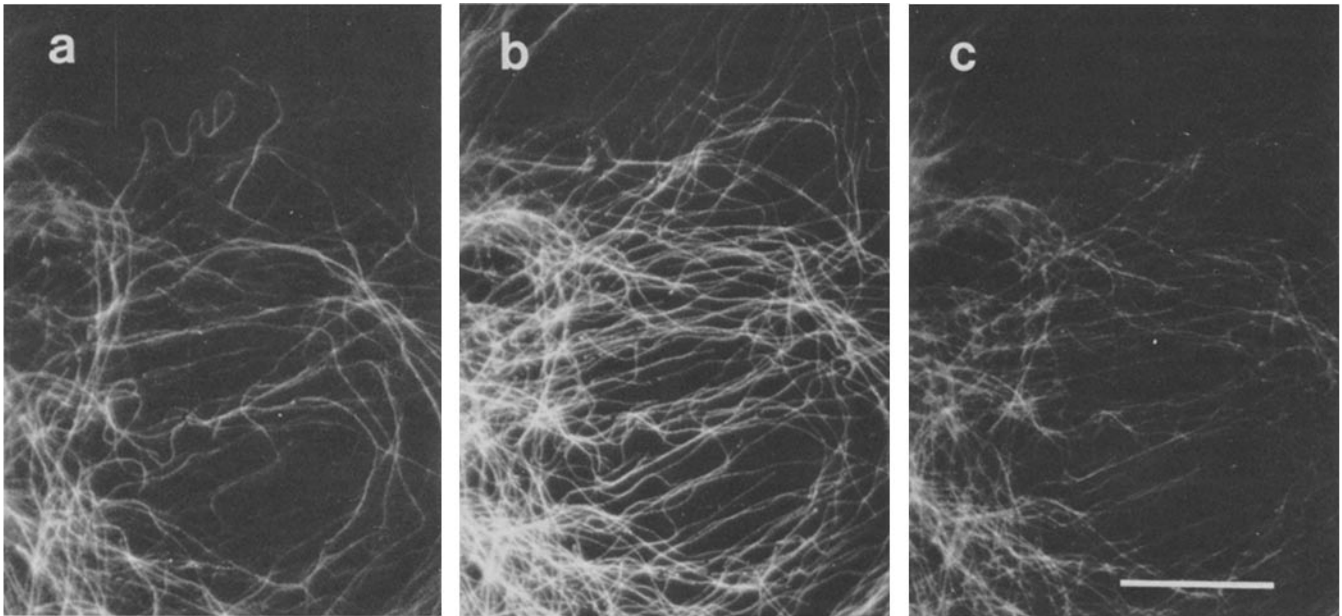

FIGURE 3 Detail of Ab 1-6.1 stained microtubule subset compared with deliberately underexposed image of entire microtubule complex. ME cells were detergent extracted and double stained by indirect immunofluorescence with the monoclonal antitubulin Ab 1-6.1 and polyclonal rabbit antitubulin antibodies. In this case, the fluorescent second antibody combination was fluorescein-labeled goat anti-mouse and rhodamine-labeled goat anti-rabbit IgG. (*a*) Cytoplasmic microtubules stained by monoclonal anti-tubulin Ab 1-6.1; (*b*) the same cytoskeleton stained by rabbit anti-tubulin antibodies; and (*c*) underexposed image of microtubules in *b*, accentuating those microtubules stained most brightly with the rabbit antitubulin antibodies. This comparison shows that the subset stained by Ab 1-6.1 is not simply equivalent to the microtubules stained brightly by rabbit antitubulin antibodies. Bar, 20 μm. × 1,000.

two other monoclonal antitubulin antibodies (Ab 1-1.1 and Ab 1-2.3) that were derived from the same immunized mouse to stain some or all of the cytoplasmic microtubules within individual mouse embryo (ME)[1] cells. Of these three monoclonal antibodies, Ab 1-6.1 appeared to stain less than the full complement of cytoplasmic microtubules (Fig. 1). The immunofluorescent pattern of microtubules stained with Ab 1-6.1 varied somewhat from cell to cell within these heterogeneous populations of fibroblastic cells. The staining pattern shown in Fig. 1, which is characterized by a brightly stained primary cilium and a few bright microtubules in the perinuclear region, however, was commonly observed. Some brightly staining microtubules and microtubule segments were also observed far out in the cytoplasm.

To determine whether Ab 1-6.1 preferentially bound to certain microtubules within a more extensive array of cytoplasmic microtubules, we used a double label indirect immunofluorescence paradigm to directly compare the monoclonal antibody staining pattern with that of total microtubules in the same cell. For these comparisons, affinity-purified polyclonal rabbit antitubulin antibodies were used to visualize the entire cytoplasmic microtubule complex. As shown in Fig. 2, all of the microtubules stained by Ab 1-6.1 were co-stained by the polyclonal antibody, but a number of microtubules stained by the polyclonal rabbit antibody were not stained with Ab 1-6.1 (compare *2a1* and *2a2*). This demonstrates that Ab 1-6.1 only binds to a restricted subset of cytoplasmic microtubules and that single cells can contain microtubules and/or microtubule segments that are antigenically dissimilar.

When either Ab 1-1.1 or Ab 1-2.3 was the primary monoclonal antibody binding reagent, the resulting patterns of monoclonal antibody-stained-microtubules were indistinguishable from the patterns of microtubules stained by the rabbit antibodies (Fig. 2, *b* and *c*). This identity of staining patterns was not due to cross reactivity between the two fluorescently labeled second antibody reagents (Fig. 2*d*). Since Ab 1-2.3 bound only to the alpha tubulin subunit, these results confirm the presence of alpha tubulin in all of the microtubules. Similar uniform staining of microtubules has been reported by Gozes and Barnstable (15) for two monoclonal antibodies to the beta subunit of tubulin. Since monoclonal antibodies to both alpha and beta tubulin could stain microtubules uniformly, the subset specificity of Ab 1-6.1 is not simply a distinction between alpha and beta subunits.

Characterization of Ab 1-6.1 Binding

To assure that the observed microtubule subset staining by Ab 1-6.1 actually represents the distribution of a unique antigenic class of microtubules, we have carried out a number of control experiments to demonstrate that this immunofluorescent observation was not caused by the procedures themselves. As shown in Fig. 3, the subset pattern of microtubules stained by Ab 1-6.1 is not simply equivalent to those stained most brightly by rabbit polyclonal antibodies. Although, Ab 1-6.1 often appeared to stain the very brightly stained putative microtubule bundles, it may have been staining only a subset of the microtubules within these bundles. It should also be noted that some of the microtubules most brightly stained by Ab 1-6.1 appear to be only lightly stained by the polyclonal antibody.

This differential staining property of Ab 1-6.1 was not due to competition with the rabbit antibodies because sequential

[1] Abbreviation used in this paper: ME, mouse embryo.

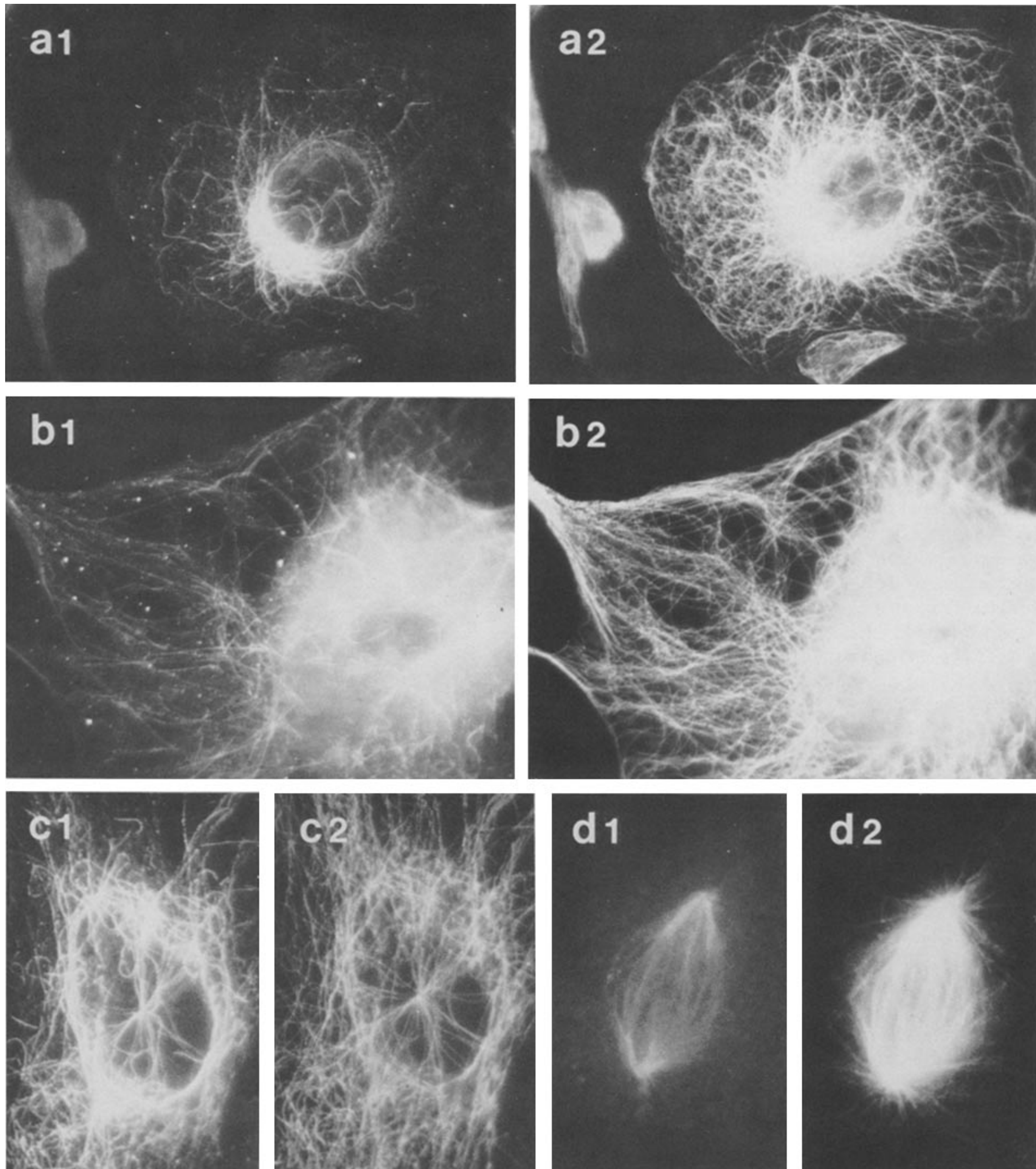

FIGURE 4 Antigenic subsets of microtubules and microtubule segments in mouse and human cells. Cytoplasmic microtubules of mouse and human cells were double stained using monoclonal Ab 1-6.1 in combination with rabbit antitubulin antibodies. As in Fig. 2, the first of each pair of fluorescence micrographs shows the pattern of bound Ab 1-6.1 (rhodamine), whereas the second shows the corresponding pattern of the cytoplasmic microtubule complex, as visualized with rabbit polyclonal antitubulin antibodies (fluorescein) within the same cells. (*a*) Detergent-extracted human foreskin fibroblast; (*b*) whole formaldehyde-fixed and acetone permeabilized ME cell; (*c*) detail of ME cytoskeleton showing microtubule organizing center; and (*d*) detergent-extracted mitotic ME cell. × 700 (*a* and *b*); × 1,000 (*c*); × 1,600 (*d*).

primary incubations first with Ab 1-6.1 followed by rabbit antibodies produced similar results. Furthermore, staining by 1-6.1 in the absence of rabbit antibodies gave similar patterns (Fig. 1). The differential staining patterns were also observed when we increased the concentration of Ab 1-6.1 ascites serum tenfold and increased the incubation period with Ab 1-6.1 from 60 to 150 min. Differential patterns were observed using second antibody reagents from several suppliers, and with the anti-mouse antibody labeled with either rhodamine (e.g., Fig. 2) or fluorescein (Fig. 3). The Ab 1-6.1 subset staining was also observed with cells and/or cytoskeletons fixed by any of several procedures, including (*a*) whole cells fixed by 4% formaldehyde and then permeabilized by extraction with either acetone, methanol, or detergent (Triton X-100); (*b*) whole cells fixed directly by −20°C acetone or methanol; (*c*) cytoskeletons fixed by 4% formaldehyde and then extracted

by −20°C acetone or methanol; (*d*) cytoskeletons fixed by 4% formaldehyde alone; or (*e*) cytoskeletons immersed directly in −20°C methanol.

Several other cell types have been examined by the double label immunofluorescence procedure to determine whether they also contain antigenic subsets of microtubules detectable with Ab 1-6.1. Human foreskin fibroblastic cells also contain a subset of microtubules that bound Ab 1-6.1 (Fig. 4*a*). Such subsets have also been detected in mouse brain cells in culture, in human lung cells in culture, and in chick embryo fibroblasts in culture. In agreement with our previous results (2), chick embryo cells possess an extensive subset of Ab 1-6.1 staining microtubules, whereas PtK_2 cells do not contain any microtubules that are stained with Ab 1-6.1. The observed differential staining pattern was not induced by the process of detergent extraction in stabilizing conditions, because similar staining patterns were observed in whole, formaldehyde-fixed cells (Fig. 4*b*).

As shown in Fig. 4*c*, some but not all of the stained microtubules or microtubule segments appeared contiguous with a conventional microtubule organizing center. It should also be noted that microtubules of both antigenic types appear to have emanated from the same organizing center (Fig. 4*c*). In mitotic cells, Ab 1-6.1 stained spindle microtubules. When such cells were in anaphase (Fig. 4*d*) it was evident that Ab 1-6.1 stained both kinetochore and nonkinetochore microtubules. It is not clear, however, whether this staining represents all or only some of the spindle microtubules. Aster microtubules do not appear to have been stained by Ab 1-6.1 in the cell shown in Fig. 4*d*, although in some cells, we have noted the staining of individual aster microtubules by this antibody.

Ab 1-6.1 Staining of Modified Cytoplasmic Microtubules

To further characterize the physical nature of the antigenic differences between Ab 1-6.1 positive and negative microtubules, we developed procedures to stabilize microtubules without fixation so that they might be modified in situ prior to antibody binding. Taxol is a macrocyclic drug that stabilizes microtubules (21) and enhances microtubule assembly (20) even at low temperature (25). As shown in Fig. 5, following detergent extraction in stabilizing buffer, microtubules can be stabilized with taxol and visualized by immunofluorescence without fixation. As shown, the microtubules appear comparable with those seen in fixed cytoskeletons when stained with rabbit polyclonal antibody (Fig. 5*b*). In contrast, no microtubules were brightly stained by Ab 1-6.1 (Fig. 5*a*). This result indicates that the Ab 1-6.1 epitope on tubulin is unavailable for binding in taxol-stabilized microtubules. Taxol may interfere directly with Ab 1-6.1 binding because the drug binding site is proximate to the Ab 1-6.1 epitope. Alternatively, the Ab 1-6.1 epitope may be cryptic in tightly organized microtubules and only becomes available following fixation. The taxol inhibition of Ab 1-6.1 subset staining appears to be reversible in that if taxol stabilized microtubules were fixed and methanol extracted prior to immunofluorescence the subset pattern was again evident. Therefore, taxol can be used to stabilize microtubules during treatments designed to physically or chemically modify cytoskeletons as a means of defining the basis for the subset specificity of Ab 1-6.1 binding.

A differential staining pattern could arise from a selective interaction of some microtubules with proteins, thereby blocking access to the specific epitope. Vallee (26) has demonstrated that microtubule-associated proteins can be extracted by 0.35 M NaCl from taxol-stabilized microtubules assembled in vitro. We used taxol to stabilize microtubules within detergent-extracted cytoskeletons and then extracted the cytoskeletons with 0.5 or 1.0 M NaCl prior to fixation. As shown in Fig. 6*a*, cytoplasmic microtubules remain after this treatment and, more importantly, the differential staining of only a subset of them is unchanged. Although the entire complement of proteins associated with microtubules in these cytoskeletons, as well as their behavior during salt extraction, remains to be defined, these results indicate that the inability of Ab 1-6.1 to bind to many microtubules does not appear to be due to the presence of readily-extractable associated proteins.

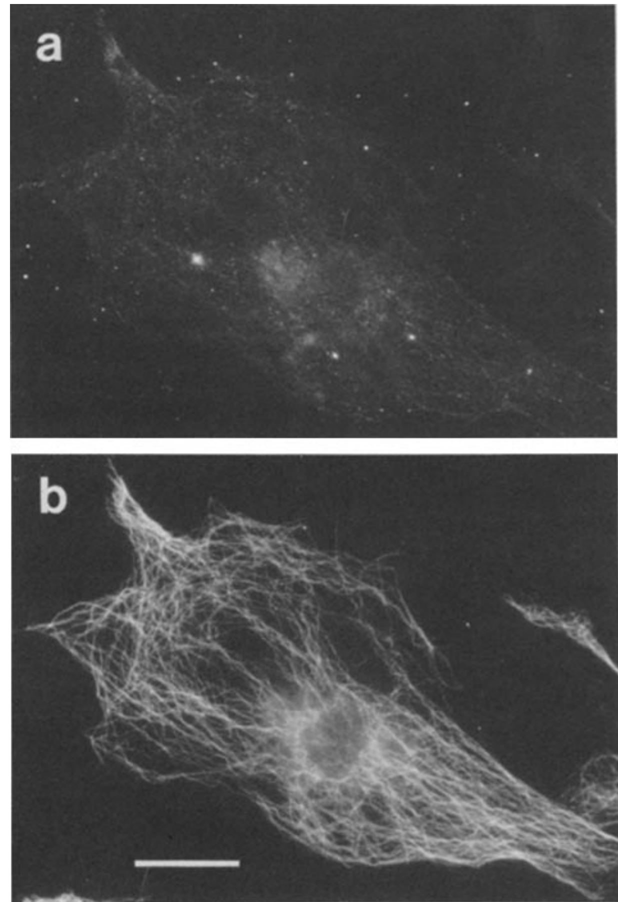

FIGURE 5 Use of taxol as stabilizing agent for indirect immunofluorescence staining of unfixed cytoplasmic microtubules. ME cells were extracted with 0.5% Triton X-100 in microtubule stabilizing buffer (MTSB-aprotinin) and then incubated for 2 min in MTSB-aprotinin containing taxol (10 μg/ml). The taxol-stabilized cytoskeletons were then subjected to double label immunofluorescence staining with monoclonal Ab 1-6.1 (*a*) and rabbit antitubulin antibodies (*b*) in the standard buffers (see Materials and Methods) supplemented with aprotinin and taxol (2 μg/ml). This experiment demonstrates that microtubules can be stabilized and visualized in unfixed cells. The lack of binding of Ab 1-6.1 to these taxol-stabilized microtubules suggests that taxol may mask the Ab 1-6.1 epitope. Bar, 20 μm. × 700.

Alpha tubulin subunits are subject to enzymatic post-translational addition and removal of tyrosine (or phenylalanine) to the carboxyl terminus in vivo (23, 24). It thus seemed

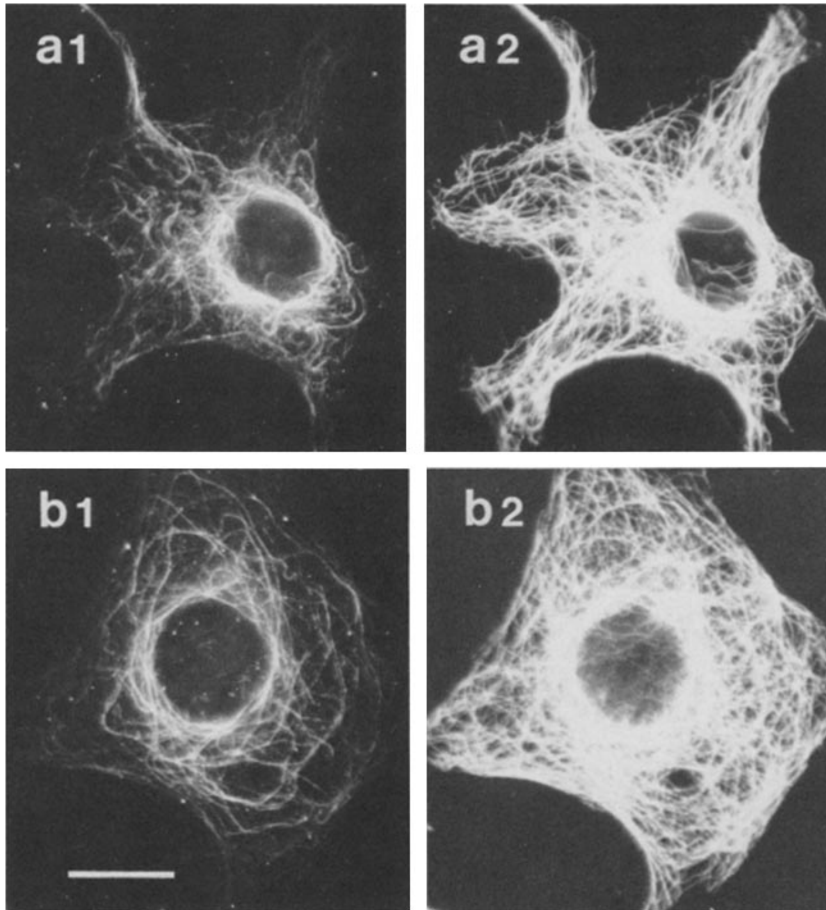

FIGURE 6 Effect of salt extraction or carboxypeptidase A digestion of cytoskeletal microtubules upon antigenic microtubule subsets. (*a*) Human foreskin cells were detergent extracted and taxol stabilized as in Fig. 5. The cytoskeletons were extracted with 1.0 M NaCl in 0.1 M PIPES, pH 6.9, 1 mM EGTA, 2 μM taxol (supplemented with aprotinin), and then rinsed in the same buffer without NaCl prior to formaldehyde fixation and acetone extraction. The salt-extracted cytoskeletons were then double stained with monoclonal Ab 1-6.1 and rabbit antitubulin antibodies as in Fig. 2; (*b*) ME cells were detergent extracted and taxol stabilized as above. The stabilized cytoskeletons were digested with pancreatic carboxypeptidase A (10 μg/ml) for 5 min at room temperature in 0.1 M PIPES, pH 6.9, 2 mM $ZnCl_2$, 2 μM taxol, prior to formaldehyde fixation and acetone extraction. The carboxypeptidase A-digested cytoskeletons were then double stained as above. *a1* and *b1* show patterns of microtubules stained by monoclonal Ab 1-6.1, whereas *a2* and *b2* show the corresponding patterns of microtubules stained by rabbit antitubulin antibodies in the same cells. Bar, 20 μm. × 700.

possible that the presence or absence of a carboxyl terminal tyrosine might influence Ab 1-6.1 binding. Kumar and Flavin have reported that tyrosine can be removed from taxol-stabilized microtubules by carboxypeptidase (18). We therefore treated taxol-stabilized cytoskeletons with carboxypeptidase A (24) and then examined the pattern of subsequent Ab 1-6.1 immunofluorescent staining. As shown in Fig. 6*b*, this treatment has no apparent effect on the differential staining by Ab 1-6.1. Wehland et al. (27) have recently demonstrated that carboxypeptidase A digestion of fixed cytoskeletons under similar conditions abolished the binding of a monoclonal antibody that recognized only the tyrosinated form of α-tubulin. In the absence of carboxypeptidase treatment, this antibody stains all cytoplasmic microtubules rather than a subset (27). Taken together, these results suggest that the subset specificity of Ab 1-6.1 is not related to the presence or absence of terminal tyrosines.

Correlation of Antigenic and Physiological Subsets of Cytoplasmic Microtubules

Cytoplasmic microtubules have previously been shown to be heterogeneous with regard to stability to several microtubule-depolymerizing conditions (3). We have examined the antigenic subsets of microtubules within cells pretreated by colcemid, low temperature, or hypotonic culture medium (Fig. 7). When ME cells were exposed to 5 μM colcemid for 1 to 2 h (at 37°C) prior to detergent extraction and double label immunofluorescence staining, most of the resulting cytoskeletons had no microtubules or microtubule-like structures that could be detected by either rabbit antitubulin antibodies or Ab 1-6.1 (not shown). However, a few microtubules or microtubule fragments remained in some cytoskeletons, all of which were stained by both Ab 1-6.1 and the polyclonal rabbit antitubulin antibodies (Fig. 7*a*). Even in cytoskeletons, such as the one pictured in 7*a*, which retained a complex array of colcemid-resistant microtubules, virtually all of the resistant microtubules were Ab 1-6.1 positive. This result demonstrates that the differential staining patterns of Ab 1-6.1 detect physiologically different microtubules that exist in single cells prior to extraction for fixation.

As shown in Fig. 7, *b* and *c*, not all populations of physiologically stable microtubules are characterized by a single antigenic type of microtubule defined by Ab 1-6.1. Two other populations of stable microtubules, those remaining after treatment with low temperatures (50 min at 4°C) or a 15-min incubation at 37°C with hypotonic medium (11) have been shown to contain microtubules of both antigenic types (Fig. 7, *b* and *c*). The patterns of the two antigenic microtubule subsets within these cytoskeletons is quite pronounced as a result of the simplified microtubule pattern in these treated cells. It may be of interest that small aster-like structures (Fig. 7*b1*), which may represent tubulin newly polymerized as the chilled cells are immersed in warm lysis buffer, are of the Ab 1-6.1 negative antigenic type. Further experiments are underway to determine whether a postassembly modification of microtubule subunits might be required for Ab 1-6.1 binding.

These results demonstrate that within single cells there is a highly segregated presentation of certain α-tubulin epitopes that can be detected by indirect immunofluorescence. This suggests that mechanisms must exist within the cell for either differential assembly or postassembly modification of micro-

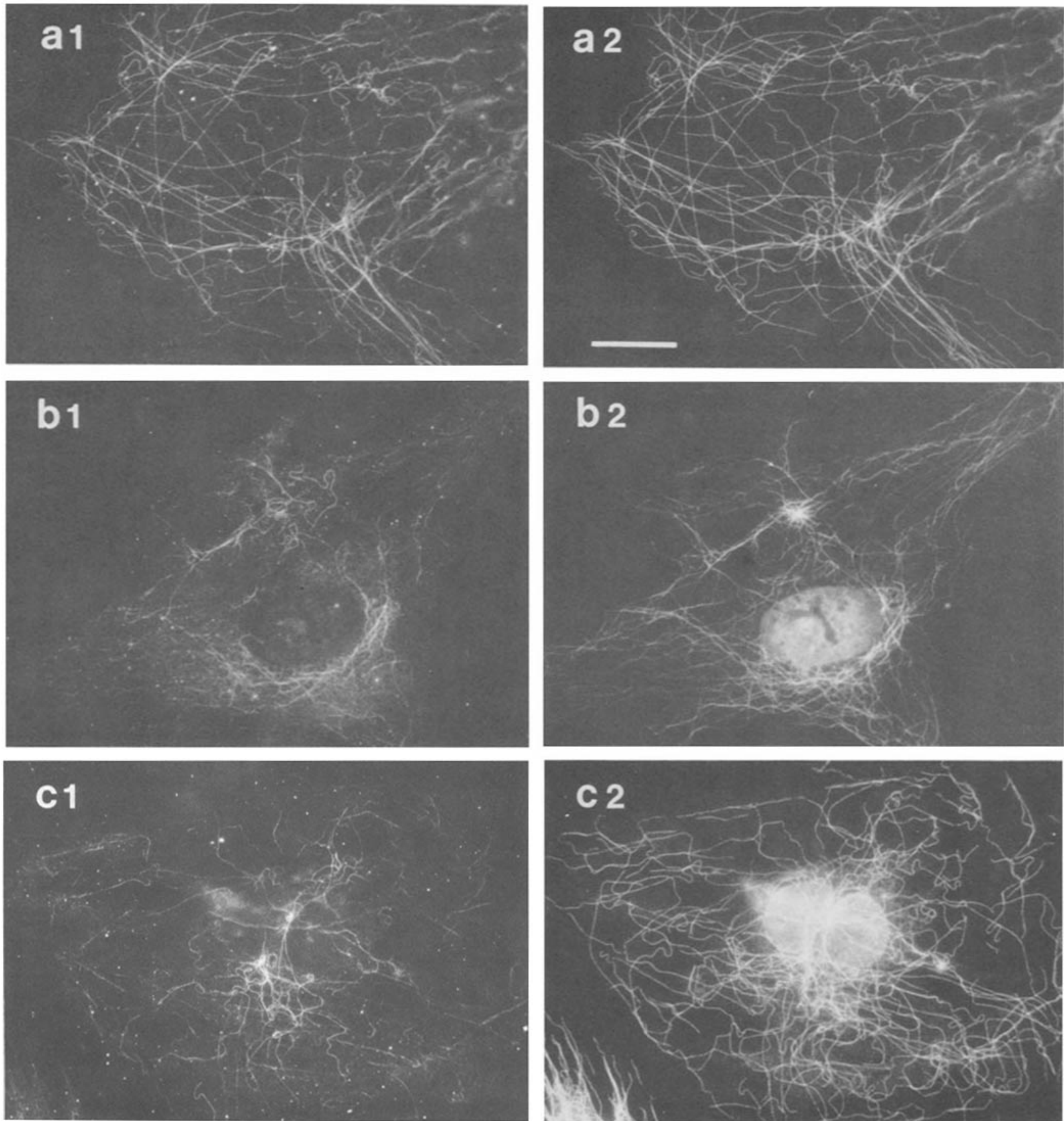

FIGURE 7 Antigenic microtubule subsets in cells pretreated to depolymerize some cytoplasmic microtubules. ME cells were subjected to several different treatments to induce some microtubule disassembly prior to double immunofluorescence staining with monoclonal Ab 1-6.1 and rabbit antitubulin antibodies. (*a*) Cells were cultured in medium containing 5 μM colcemid for 2 h prior to detergent extraction, fixation, and immunofluorescence staining; (*b*) cells were rinsed with 0°C medium and maintained in the cold for 50 min prior to detergent extraction, fixation, and immunofluorescence staining; and (*c*) cells were pretreated with hypotonic culture medium (Dulbecco Voght-modified Eagle's medium diluted with 4 vol water) for 15 min prior to formaldehyde fixation of the whole cells, followed by methanol extraction, and immunofluorescence staining. The first of each pair of fluorescence micrographs shows microtubules stained by Ab 1-6.1, whereas the second shows the corresponding pattern of microtubules stained by rabbit antitubulin antibodies in the same cells. Note that all of the microtubules remaining after the colcemid treatment bound Ab 1-6.1, whereas that was not the case following treatment with cold or hypotonic medium. Bar, 20 μm. × 700.

tubules or microtubule segments expressing different antigenic determinants. Moreover, the correlation of these antigenic differences with the physical stability of microtubules that follow drug treatment suggests that these antigenic differences might reflect an organization of the cytoskeleton into functionally distinct subsets of microtubules. Crossin and Carney (9, 10) have previously presented evidence that the mitogenic actions of thrombin and epidermal growth factor

upon such fibroblastic cells may require an early transient depolymerization of microtubules. It is intriguing to speculate that such regulatory events might involve only specific subsets of microtubules. The development of monoclonal antibodies that can distinguish different microtubules and perhaps distinguish individual tubulin variants should prove particularly useful in dissecting the functional organization of the cytoskeleton.

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