ABSTRACT
We have studied the distribution of myosin and tubulin molecules inside the same tissue culture cells by using two antibodies labeled with contrasting fluorochromes. Antimyosin raised against human platelet myosin was labeled with rhodamine. Antitubulin raised against sea urchin vinblastine-induced tubulin crystals was labeled with fluorescein. The two antibodies stained entirely different structures inside the same flat interphase cell: antimyosin bound to stress fibers and antitubulin bound to thin, wavy fibers thought to be individual microtubules. Compact interphase cells stained diffusely with both antibodies. From prophase through early anaphase both antibodies stained the mitotic spindle, although the fluorescence contrast between the spindle and the cytoplasm was much higher with antitubulin than with antimyosin. From anaphase through telophase, strong antimyosin staining occurred in the cleavage furrow, while antitubulin stained the region between the separated chromosomes. This study establishes the feasibility of high-resolution fluorescent antibody localization of pairs of motility proteins in the cytoplasm of single cells, an approach which will make it possible to map out the sites of the various contractile protein interactions in situ.

KEY WORDS double antibody staining • antimyosin • antitubulin • spindle • cleavage furrow

In the structural analysis of cellular motile systems, it has become essential to know how the component molecules are arranged relative to one another inside cells to confirm that interactions demonstrated with purified proteins are actually possible in situ. In previous studies, single proteins have been localized by using myosin fragments to identify actin (15, 22) and fluorescent antibodies for actin and other proteins (2, 10, 16, 17, 18, 29) thought to play important roles in cell motility. Comparison of the distribution of the various proteins in different cells has led to inferences regarding their interactions in the cell. It remains important to demonstrate two or more of these proteins simultaneously in a single cell, and to establish their spatial relationships and, hopefully, the sites of their interaction.

Previous work established the feasibility of staining biological materials simultaneously with two antibodies labeled with contrasting fluorochromes. In most cases the object was to identify two different cell types within a tissue section or a cell smear (6, 13, 14, 20). The fluorescent double-labeling technique was also applied to the analysis of macromolecular rearrangement on the cell surface (7, 8). The goals of the present work were (a) to perfect methods for studying the distribution of two different motility proteins inside the
Rhodamine-labeled Antimyosin IgG

Preparation of Antimyosin Antiserum and Rhodamine-labeled Antimyosin IgG

We have described (10) the purity of the myosin preparations used to immunize rabbits, the specificity of the antimyosin antiserum and the procedure used to make rhodamine-labeled antimyosin immunoglobulin G (IgG). In this study we used rhodamine-labeled IgG isolated from the antiserum against the rod portion of human platelet myosin (rabbit no. 8 described in reference 10).

Preparation of Antitubulin Antiserum

ANTIGEN: We isolated tubulin from unfertilized eggs of sea urchin, Strongylocentrotus purpuratus, in the form of a crystal induced by treatment with vinblastine sulfate (1, 4, 19). The gametes were obtained from mature animals by injecting 0.6 M KCl solution into the body cavity. The eggs were washed three times in artificial seawater prepared according to the formulation of the Woods Hole Marine Biological Laboratory (Woods Hole, Mass.). Up to 2 ml of packed eggs were suspended in 20 ml of artificial seawater containing 0.1 mM vinblastine sulfate (Velban, Eli Lilly and Co., Indianapolis, Ind.) and 0.1 mM Colcemid. Colcemid was included to increase the rate of induction and the yield of crystals (25). The cells were incubated with a constant agitation at 22°C for 12-18 h in a Petri dish covered with aluminum foil. Vinblastine-induced crystals were isolated by the method described by Bryan (4) with a slight modification. One milliliter of packed eggs was collected by hand centrifugation and suspended in 10 ml of 1 M urea containing 5 mM Tris-HCl (pH 8.5) for 2 min. They were pelleted quickly by hand centrifugation, and the supernate was discarded. The eggs, which were quite fragile and sticky, were carefully washed in fresh artificial seawater. They were then suspended and lysed in 10 ml of the stabilizing medium containing 100 mM KCl, 1 mM MgCl₂, 0.1% Triton X-100, and 10 mM Tris-HCl at pH 7.5. Vigorous pipetting for at least 2 min was required to lyse the eggs completely. The egg lysate was then centrifuged at 1,500 g for 3 min and the supernate discarded. The pellet was resuspended in the stabilizing medium, vigorously pipetted, and centrifuged at the same speed for 3 min. The last step was repeated one more time and the final pellet, purified vinblastine-induced tubulin crystals, was obtained. The isolated crystals were examined by phase-contrast microscopy, and preparations contaminated with any cellular debris were rejected. Clean isolates were pooled and stored in the detergent-free stabilizing medium containing 0.2% Na₂O at 0°C for up to 2.5 mo.

IMMUNIZATION: We used the methods for immunization and bleeding described in our previous paper (10). After collecting about 50 ml of preimmune serum from each of four White New Zealand rabbits, we immunized each rabbit with 250 μg of protein in complete Freund's adjuvant and then boosted once with the same amount of antigen in incomplete Freund's adjuvant. The serum was kept frozen at −20°C until use.

Testing of Antitubulin Antisera

Serum from each rabbit was tested by double diffusion in agar and immunoelectrophoresis as described previously (10), except that we employed 100 mM Veronal buffer at pH 8.6 in place of pyrophosphate buffer. The test antigens included purified sea urchin tubulin crystals, solubilized by freezing and thawing in 100 mM piperazine-N,N'-bis[2-ethane sulfonic acid] (PIPES) and 1 mM ethylene glycol-bis(β-aminoethyl ether)N,N',N',N"-tetraacetate (EGTA) at pH 6.9, purified pig brain tubulin, generously provided by Dr. R. Linck (Department of Anatomy, Harvard Medical School) and crude tissue and cell extracts. A crude brain extract containing tubulin was prepared from 17-day chick brain by homogenizing in 100 mM PIPES and 1 mM EGTA and centrifuging at 100,000 g for 60 min at 4°C. HeLa cell extracts were made by harvesting cells grown in tissue culture flasks in 5 mM EDTA in Ca-Mg-free Hanks' solution, washing once in this solution and freeze-thawing the pellet. An equal volume of twice concentrated PIPES-EGTA buffer was added, and the cell suspension was briefly sonicated. After 1-h extraction on ice, the extract was cleared by centrifugation at the maximum speed of a clinical centrifuge for 10 min at 4°C.

Preparation of Fluorescein-Labeled IgG from Antitubulin Antiserum

We selected one of the four rabbit antisera against tubulin, conjugated its IgG with fluorescein isothiocyanate, and fractionated the reaction products on O-(diethylaminoethyl) cellulose (DEAE-cellulose) (6, 10). The protein to dye ratios were 1:2 for rhodamine antimyosin and 1:1 for fluorescein antitubulin.
Preparation of Tissue Cells for Antibody Staining

Human tissue culture cells, HeLa, WI 38 and Enson and rat kangaroo cells PtK-2, were grown on 18 x 18 microscope cover glasses as previously described (10). The salamander lung tissue cultures were made in Rose chambers by Dr. Y. Ohnuki (Pasadena Foundation for Medical Research, Pasadena, Calif.) and shipped by air to Boston. All these cells were prepared for antibody staining by fixation in acetone and formalin according to a method described previously (23).

Double Staining of Cells with Antimyosin and Antitubulin

Cells were stained by inverting the cover glass onto 60 µl of solution containing 30-40 µg of fluorescein-labeled antitubulin IgG and 4-8 µg of rhodamine-labeled antimyosin IgG. The staining was carried out in a moist chamber at 37°C for 30-45 min. After staining, the cells were washed in phosphate-buffered saline and mounted in 90% glycerol in the saline (10).

The following controls were done: (a) To examine whether or not there is autofluorescence, we observed fixed but unstained cells with filter combinations appropriate to detect fluorescein and rhodamine. (b) To test for the presence of nonspecific staining, we stained cells with labeled IgG from an unimmunized animal. (c) To examine the immunological specificity of the staining pattern, we first blocked antibody binding sites in the cell with unlabeled antibodies and then stained with labeled antibodies as described in Fig. 8. We also stained cells with absorbed labeled IgG preparations. The rhodamine-labeled antimyosin IgG was absorbed by means of affinity chromatography (10). The fluorescein-labeled antitubulin was absorbed with three types of antigen: isolated sea urchin sperm tail axonemes, purified calf brain microtubule protein, and vinblastine-induced sea urchin egg tubulin crystals. 50 µg of labeled antitubulin IgG was absorbed with 5-20 µg of the antigens for 12 h at 4°C. The supernate obtained after centrifugation at 1,500g for 10 min was used to stain cells. The axoneme preparation was a generous gift from Dr. R. Linck and purified calf brain tubulin was supplied by Linda Griffith (Department of Anatomy, Harvard Medical School).

Fluorescence Microscopy and Photomicroscopy

To differentiate the fluorescence of fluorescein (green) from that of rhodamine (red), we used the following filter combination with a Leitz Orthoplan microscope stand equipped with a Ploem vertical illuminator and a Zeiss 63 x (NA 1.4) planapo phase-contrast objective lens. The filter combination is described in the order through which the illuminating light from a Xenon lamp (XBO 150) passes. For fluorescein the combination was 3 mm BG-38 (red suppression filter), KP 500 (short wavelength pass interference filter), TK-510/515 (built in dichroic mirror and colored glass barrier filter), and S-525 (narrow band pass interference filter). The last filter is necessary to eliminate the transmission of any rhodamine fluorescence. For rhodamine, we used a 3-mm BG-38, KP 560 (short wavelength pass interference filter), 2-mm BG-36 (excitation filter), TK-580 (dichroic mirror), and two K-580 (colored glass barrier filters). Although this rhodamine filter combination is satisfactory for rhodamine alone, the excitation filters transmit short wavelength light which excites fluorescein and the barrier filters transmit that small fraction of the fluorescein emission spectrum above 580 nm. Consequently, when viewing rhodamine in the presence of high intensity fluorescein emission, we included a K 530 colored glass filter in the exciting beam to reduce the intensity of the exciting light below 530 nm. This additional filter eliminated any detectable fluorescein signal when observing rhodamine. Due to the rapid bleaching of fluorescein by the exciting beam, it is necessary to observe and photograph the fluorescein signal before the rhodamine signal. Even with this precaution, the fluorescein is much more difficult to record than the rhodamine fluorescence.

RESULTS

Immunology

TUBULIN ANTIGEN: We purified sea urchin egg tubulin for immunization in the form of vinblastine-induced crystals. By gel electrophoresis in sodium dodecyl sulfate (SDS) the tubulin preparation consists primarily of α- and β-tubulin subunits (Fig. 1). The high-molecular-weight polypeptides which are known to be present in the isolated crystals (5) comprise about 1% of the total protein. The three minor bands whose molecular weights are less than tubulin represent about 2% of the total protein. They may be break-down products of tubulin, since they increased during storage of the crystals.

TUBULIN ANTISERA: Double immunodiffusion and immunoelectrophoresis showed that the antisera reacted with tubulin from a variety of species but not with other components of crude tissue extracts (Fig. 2). In addition to the reaction with purified vinblastine crystals and crude chick brain extract shown in Fig. 2, the antisera formed a single precipitin line after double diffusion or immunoelectrophoresis against sea urchin sperm tail tubulin, pig brain tubulin, and HeLa cell extracts. The extent of the species cross-reactivity will be presented elsewhere.
Electrophoretic analysis of tubulin antigen. The polyacrylamide gel is stained with Coomassie blue and scanned at 550 nm. 97% of the total protein is α- and β-subunits of tubulin. The high-molecular-weight contaminants and low-molecular-weight polypeptides represent 1% and 2% of the total protein, respectively.

Fixation

Before staining cells with labeled antibodies, it is necessary to fix them to immobilize the cytoplasmic antigens and to make the membrane permeable to fluorescent immunoglobulins. The present fixation method minimized structural alterations of the mitotic spindle detected by a light microscopy (23). Interphase cells also maintained a lifelike morphology after being fixed in this manner (see the phase-contrast micrographs in Figs. 5 and 8).

When we analyzed the fixative and wash solutions employed here by gel electrophoresis in SDS, no myosin was detectable in any of the solutions. This rules out any myosin extraction during fixation, but leaves open the question of possible myosin redistribution within the cell. On the other hand, a small amount of protein with the electrophoretic mobilities of actin and tubulin were present in the wash solutions. Thus, some tubulin may be extracted from the cell during fixation and washing.

Since we were unable to devise a fixation procedure which immobilized myosin and tubulin without altering morphology and which preserved good antigenicity of these proteins, the present fixation was used as the best compromise for this study. Details of these fixation studies will be presented elsewhere.

Antitubulin Staining of Tissue Culture Cells

Before attempting double staining with two antibodies, we stained a variety of cells with fluorescein-antitubulin and were able to confirm the staining patterns observed previously (3, 23, 26) with indirect staining methods. In well-spread cells such as rat kangaroo PtK-2 cells, the fluorescent antibody stained thin, wavy fibers (Fig. 3a). The fibers are less than 0.4 μm wide and stain continuously along their length. The thin fibers are concentrated around the nucleus. Some fibers extend radially from the region of the nucleus toward the boundary of a cell, while others are arranged circumferentially. This arrangement creates a fluorescent network in the cytoplasm. The degree of intertwining of the fibers varies among cell types and is high in rat kangaroo cells. In salamander lung cells the intertwining is much less. Most fibers are parallel and extend from the nucleus to the cell cortex (Fig. 3b). In dividing cells the fluorescein-antitubulin is concentrated in the mitotic spindle. In cells with large spindles such as PtK-2 and salamander lung cells the individual spindle fibers are easily visualized and photographed (Fig. 3c).

Double Staining of Human Tissue Culture Cells with Antimyosin and Antitubulin

In the following sections we show that tubulin and myosin molecules independently change their distribution within the cytoplasm as the cell passes...
Figure 3 Fluorescence micrographs of PtK-2 (a) and salamander lung tissue culture cells (b and c) stained through the cell cycle. Only during mitosis are the two proteins concentrated together in the same region, the mitotic spindle. All of the illustrations are of HeLa cells, but similar observations were made with two other human cell lines, WI-38 and Enson.

Interphase: As shown previously (10) the antimyosin staining pattern varies from cell to cell and seems to depend on the shape of the cell. In flattened cells the antimyosin staining is usually punctate and concentrated along stress fibers (Figs. 4 and 5). In more rounded cells most of the staining is diffuse but some is concentrated at the periphery (Fig. 4 and Fig. 11 in reference 10). This antimyosin staining near the cell surface is concentrated in the cortex rather than outside the cell, as we found no surface staining of HeLa cells or platelets with the antimyosin (10, 21). In most cases, areas with ruffled membranes do not stain heavily as do other regions of the cortex.

Antitubulin staining is concentrated in the perinuclear region of all HeLa cells. A few cells have a distinct network of intertwined, radially oriented fibers (Fig. 4), similar to the networks in PtK-2 cells. Most HeLa cells have either a poorly resolved fiber network (Fig. 5) or diffusely stained cytoplasm (Fig. 4). In none of the cells did we see an intense staining of the cell periphery with antitubulin.

Comparison of the antimyosin and antitubulin staining patterns within the same cell show that they are entirely different (Figs. 4 and 5). This indicates that we can detect the two fluorescent antibodies completely independently inside the same cell. For example, antimyosin alone stains stress fibers while only antitubulin stains the perinuclear region. Stress fibers and antitubulin staining fibers are located at different depths in the cytoplasm. Stress fibers are usually found near the base while antitubulin fibers are found at all levels in the cytoplasm.

In general, the cells with diffuse antimyosin staining have diffuse antitubulin staining. In cells with stress fibers, the antitubulin fiber networks vary from elaborate to poorly developed. Thus, the degree of order in the two systems is not necessarily parallel in individual cells, although with fluorescein antitubulin. Thin, wavy fibers in the cytoplasm form a network pattern in PtK-2 (a) while they are more parallel and arranged radially in salamander lung cells (b). A metaphase spindle stains brightly revealing spindle fibers (c). Bars, 20 μm.
Fluorescence micrographs of HeLa cells stained simultaneously with fluorescein antitubulin (a) and rhodamine antimyosin (b). Antitubulin stains thin wavy fibers and the perinuclear region (a) and antimyosin stains stress fibers and cortex of some compact cells (b). Two cells on the left side of the micrographs are recently divided daughter cells with a cytoplasmic bridge containing the midbody stained brightly with antitubulin (a) but not with antimyosin (b). Antimyosin staining in these cells is diffuse, and their morphology is that of the motile type (b). Scale, 1 division = 10 μm.
Figure 5. Micrographs of HeLa cells stained simultaneously with fluorescein antitubulin (b) and rhodamine antimyosin (c). The phase-contrast micrograph (a) shows the general morphology of the cells. The discrimination of the two fluorescent patterns is clearly illustrated. Scale, 1 division = 10 μm.
flattened cells with stress fibers are more likely to have antitubulin networks than cells that stain diffusely with antimyosin.

**Dividing Cells:** During cell division there are dramatic reorganizations of both myosin and tubulin inside the cells. Double antibody staining shows that both antigens are concentrated in the mitotic spindle, while they are concentrated in different regions of the cytoplasm during cytokinesis.

In prophase the whole cytoplasm, exclusive of the chromosomes, stains with both antimyosin and antitubulin, and both antibodies concentrate in one region near the chromosomes (Figs. 6a–c). The antitubulin is concentrated in this region to a greater extent than the antimyosin. There is no phase-dense structure corresponding to this intensely stained area.

During metaphase and early anaphase the mitotic spindle stains with both antibodies (Figs. 6d–o), although there are distinct differences in the patterns and contrast revealed by the two antibodies. As in the case of large spindles (Fig. 3b), HeLa spindles stained with antitubulin consist of

![Micrographs of dividing HeLa cells](image_url)

**Figure 6** Micrographs of dividing HeLa cells (c, f, i, l, and o) stained simultaneously with fluorescein antitubulin (a, d, g, j, and m) and rhodamine antimyosin (b, e, h, k, and n). One cell is in prophase (a–c), and other cells are at metaphase. Antitubulin stains organizing (a) and fully developed (d, g, j, and m) spindles. The range of contrast of the rhodamine antimyosin fluorescence between the spindle and the surrounding cytoplasm is illustrated in frames e, h, k, and n. The contrast is high in frame e, intermediate in frames h and k, and low in frame n. Note the cortical staining with antimyosin (e, h and n). Scale, 1 division = 10 μm.

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fibers connecting the chromosomes and the poles. HeLa spindles are so small that it is difficult to record this three-dimensional fibrous substructure on film (Figs. 6d, g, j, and m) although it is easily appreciated by focusing through the spindle. In contrast, antimyosin stains the same spindle diffusely, and there is no detectable internal structure (Figs. 6e, h, k, and n). In most cases the intense region of the antimyosin staining extends beyond the edges of the spindle defined by antitubulin staining. The contrast in fluorescence intensity between the spindle and the surrounding cytoplasm is high with antitubulin and is variable, but usually less striking, with antimyosin. This makes it difficult to capture on film what is readily apparent by microscope examination. In Fig. 6, we present micrographs illustrating the range of antimyosin fluorescence contrast that can be recorded between the spindle and the surrounding cytoplasm. The contrast ranges from striking (Fig. 6e) to slight (Fig. 6n).

Late in anaphase the area between the chromosomes and the poles and the polar regions continue to stain strongly with antitubulin (Fig. 7).

**Figure 7** Micrographs of dividing HeLa cells (c, f, i, l, and o) stained simultaneously with fluorescein antitubulin (a, d, g, j, and m) and rhodamine antimyosin (b, e, h, k, and n). One cell is in late anaphase (a–c), and the rest are in telophase. Antitubulin staining shows changing patterns of tubulin distribution in late stages of cell division while antimyosin staining shows the presence of myosin in the cleavage furrow during cytokinesis. Note the cortical staining in some cells with antimyosin (b, h, and n). Scale, 1 division = 10 μm.
7a), and, while antimyosin also stains these regions, the intensity of the fluorescence is almost the same as that of the neighboring cytoplasm (Fig. 7b). The brightest antimyosin staining in late anaphase surrounds the equator near the cell surface. This region will become the cleavage furrow. During cytokinesis the staining patterns of the two antibodies are clearly separated, with antitubulin staining fibrous structures running between the poles (Figs. 7d, g, j, and m) and antimyosin staining the cleavage furrow (Figs. 7e, h, k, and n). As cleavage progresses, the red ring and the furrow close around the green midbody. When the two daughter cells flatten and move apart, they remain connected by a cytoplasmic bridge containing the midbody which stains with antitubulin (Fig. 4a). The remainder of the cytoplasm is diffusely stained with antitubulin. Antimyosin stains throughout the cytoplasm of these spreading cells, although a few short stress fibers may also be present (Fig. 4b).

Throughout cell division some of the antimyosin staining is concentrated in small patches in the cortex of many, but not all, HeLa cells (Figs. 6e, h, and n; Figs. 7b, h, and n).

**Controls**

The fluorescence intensity in the various controls ranged from very faint to undetectable. When there is a fluorescent signal, it is so faint that it cannot be recorded on the film. Fixed but unstained cells show no autofluorescence (data not shown). Cells stained with fluorescein-labeled unimmunized rabbit IgG show no fluorescence (Figs. 8a and b). Cells treated with unlabelled specific antisera before staining with labeled antibodies show very weak fluorescence, but the level of fluorescence is too low to record it on the film (Figs. 8c, d, and e). Unlabeled preimmune sera did not block the antimyosin or antitubulin staining. Absorbed antimyosin and antitubulin are ineffective in staining cells (Figs. 8f, g, and h). These results establish the immunological specificity of the staining patterns.

**DISCUSSION**

This study establishes the feasibility of determining the spatial arrangement of a pair of cytoplasmic motility proteins inside a single cell by using two antibodies labeled with contrasting fluorochromes and focuses on the relationship of myosin and tubulin during cell division. Subsequent work has shown that the technique described here can be used to localize myosin and α-actinin or tropomyosin and α-actinin simultaneously in chick primary culture cells and PtK-2 cells (11). This same double staining method was also used to demonstrate redistribution of myosin associated with Ig capping in mouse lymphocytes (24). It is anticipated that further studies employing pairs of antibodies against various proteins will enable us to map out their relative positions and hence their sites of interaction in the cytoplasm. The first section of the discussion deals with technical details and the second with the interpretation and biological significance of the staining patterns.

**Methods**

Tubulin and myosin proved to be ideal proteins with which to test the feasibility of high-resolution double antibody staining within the cytoplasm of single cells, because the two proteins have distinctive distributions in flattened interphase cells. The success of the method depends on the specificity of the two antibodies and the use of appropriate fluorochromes and filters to detect the two antibodies independently.

**ANTIBODY SPECIFICITY:** The two antibodies precipitate with their eliciting antigens, and the controls presented here and in our previous paper on myosin antibodies (10) rule out most of the artifacts likely to produce nonspecific staining of other cell components. As discussed in detail previously (10), these controls leave open the possibility that some of the staining is due to the presence of a low concentration of antibody against a minor contaminant in the original antigens. However, even if present, this type of nonspecific staining would make, at the most, a small contribution to the total fluorescence intensity in any micrograph.

**FLUOROCHROMES AND FILTERS:** The pairs of fluorescence micrographs of doubly stained interphase cells (Figs. 4 and 5) show that the two antibodies are detected without interference from one another. No antimyosin signal is detected with the fluorescein filters and no antitubulin signal is detected with the rhodamine filters. The rapid bleaching of fluorescein and the necessity of using a narrow band pass barrier filter made the photography of the antitubulin images much more difficult than that of the rhodamine-antimyosin images. To compensate in part, we
FIGURE 8 Staining controls. The field shown in (a) is stained with fluorescein-labeled IgG from an unimmunized rabbit and observed with a fluorescence microscope (b). No staining is observed except in some cell debris. The field shown in (c) is stained with fluorescein-labeled antitubulin (d) and rhodamine-labeled antimyosin (e) after the treatment with a one to one mixture of unlabeled antisera against tubulin and myosin. Most of the antibody binding sites are blocked by the unlabeled antibodies. The field shown in (f) is stained with fluorescein-labeled antitubulin absorbed with 8 μg of sperm tail axoneme (g) and rhodamine-labeled antimyosin absorbed by affinity chromatography (h). No staining is observed. Cells stained with fluorescein antitubulin absorbed with various amounts of calf brain tubulin or vinblastine crystals were not fluorescent.

Interpretation of Staining Patterns

We have interpreted the staining patterns by assuming that fluorescence intensity is propor-

used a much higher concentration of fluorescein-antitubulin than rhodamine-antimyosin in the staining mixture.
tional to antigen concentration and that all of the antigen is retained and accessible to the antibody in the fixed cells. Work in progress indicates that none of the myosin is extracted from HeLa cells by our fixation and washing procedures, but we have not measured whether the antigenicity is preserved or whether all of the antigen molecules are accessible to the antibody.

The double staining method shows that myosin and tubulin move about in the cytoplasm independently in predictable ways as a cell passes through the cell cycle. Except for mitosis when both proteins are concentrated together in the mitotic spindle, tubulin and myosin do not appear to be parts of the same cellular structures. We consider in the following paragraphs the detailed interpretation of the staining patterns at each stage in the cell cycle.

Interphase cells can be divided into two general classes on the basis of their morphology and staining patterns. On one hand there are flat cells which spread widely on the coverslip, and on the other hand more compact cells, many of which have a motile morphology.

In the spread cells the antomyosin and antitubulin stain two morphologically distinct and spatially separated structures. Antimyosin is located in randomly oriented, straight, stress fibers. Antitubulin stains thin, wavy fibers which tend to be radially oriented. Within the limit of resolution of a light microscope, we find no morphological association of these two classes of fibers. As suggested by previous investigators (2, 26, 28), it seems possible that the fine fibers stained with antitubulin may be individual microtubules, although there is no direct proof of this point.

Both antomyosin and antitubulin stain the cytoplasm of the compact cells diffusely. The thickness of these cells and the limited resolution of the light microscope may prevent the detection of an ordered arrangement of myosin and tubulin, because either an overlapping network of fibers or a nonfibrous distribution of antigens could give the same diffuse image. In fact, the presence of stained "microtubules" near the periphery of many of these cells indicates that they may be present in the thicker regions as well. In contrast to the spread cells where it was possible to rule out extensive myosin-microtubule interactions morphologically, there could well be such interactions in the thicker regions of these cells.

During mitosis both myosin and tubulin are concentrated in the mitotic apparatus. Judging from the contrast between the cytoplasm and the mitotic spindle, the tubulin is concentrated in the spindle to a far greater extent than the myosin. Most interestingly, the tubulin and myosin have different distributions within the spindle. When focusing through the double-stained spindle, one sees green (tubulin) fibers embedded in a red (myosin) cloud. Thus, the myosin molecules seem to surround the spindle fibers, but few of them are obviously aligned along the microtubules. This eliminates the possibility that myosin is concentrated in the spindle simply because it is artificially stuck to the microtubules and suggests that there must be some other mechanism responsible for concentrating the myosin there. Finding the myosin between the microtubules also strengthens the muscle-bone analogy for describing the mitotic spindle. Whether the muscle-bone myosin-microtubule analogy extends to the level of function is, of course, an unproven, but attractive, hypothesis.

As the chromosomes move to the poles, there is a rapid redistribution of myosin from the region between the chromosomes and the poles to the region of the forming cleavage furrow. The pole-to-pole spindle fibers in the interzone between the chromosomes stain strongly with antitubulin, so that in telophase one observes an axial bundle of straight green fibers surrounded by an equatorial red ring. As cleavage progresses, the red ring and the adjacent cell surface close around the midbody. This spatial separation of the two proteins provides further evidence that tubulin and myosin do not bind readily to one another and that their distribution in the cell is controlled separately. If microtubules and contractile proteins cooperate in the cell, some other protein, perhaps actin or actin plus another protein, must link the contractile system powered by myosin to the cytoskeletal microtubules.

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