Mitosis in a Cell with Multiple Centrioles

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ABSTRACT N115 mouse neuroblastoma cells possess a large number of microtubule organizing centers (MTOCs) which can be identified ultrastructurally as single centrioles. The distribution and activity of these organizing centers can be followed through all stages of the cell cycle by labeling microtubules with anti-tubulin and chromatin with the Hoechst dye, Bisbenzimid. We have found that multiple MTOCs persist and continue to organize microtubules during mitosis. They exhibit a well-defined sequence of movements, starting from a loose cluster during interphase, proceeding to a widely and evenly dispersed arrangement in prophase, gathering into small clusters and chains during prometaphase, and residing in two ring-shaped groups at the mitotic poles during metaphase and anaphase. Despite their large number of centrioles, virutally all N115 cells show a normal bipolar mitosis, but often with unequal numbers of centrioles at the two poles. Such observations bring into question the importance of the centriole in establishing bipolar division in this cell type.

Recently several laboratories have suggested that the principal organelle responsible for microtubule organization is the microtubule organizing center or MTOC. (1, 2, 3). We have recently studied the behavior of MTOC's in mouse neuroblastoma cells (4), small polygonal cells that grow indefinitely in culture, but retain the ability to extend one or more neurites when subjected to serum starvation (5). N115 cells contain 10–20 MTOC's, which are visible as the sites of initial microtuble regrowth after drug or cold induced depolymerization (4). We have shown that cellular differentiation, as indicated by neurite outgrowth, is preceded by a rearrangement of the multiple MTOC's from a dispersed state to a single, tight cluster. Coalescence of MTOC's into a compound MTOC is followed by extension of new microtubules along the growing neurite. Recently, Sharp Osborn, and Weber (6) have shown that each of the multiple MTOC's in N115 cells and in another mouse neuroblastoma cell line is composed of a single centriole. Similar observations have also been reported by Brinkly et al. (7) on the N115 neuroblastoma line.

We wished to know how MTOC's behaved during the mitotic cycle of N115 cells. These cells provided an attractive object for study, first because they offered another series of morphogenetic events that could be correlated with MTOC behavior, and second because it was not obvious how mitosis would proceed in a cell possessing such a high number of organizing sites. A consideration of these problems in this unique cell has led us to propose a number of questions about the N115 system: (*a*) Do the multiple MTOC's persist throughout the entire N115 cell cycle? (*b*) If so, how do the MTOC's distribute at each stage of mitosis? (*c*) Do all the MTOC's function in microtubule organization during mitosis? (*d*) Do these cells organize a normal, bipolar mitotic spindle despite their multiple MTOC's? (*e*) If so, how is this accomplished?

MATERIALS AND METHODS

In most cases, multiple staining experiments were begun by growing N115 mouse neuroblastoma cells on glass cover slips. N115 cells (a gift of Dr. Marshall Nirenberg) were maintained as described previously (4). On the day before an experiment, triturated cells in 10 ml of medium were plated onto glass half cover slips (Corning 1½, 1 × 2 cm) in a 100-mm petri dish and incubated overnight.

If N115 cells on cover slips are exposed to Triton X-100 to prepare cytoskeletons, many interphase cells and virtually all mitotic cells are washed off during lysis or subsequent staining procedures. To circumvent this problem the upper and lower edges of a microscope slide were slightly raised by applying several strokes of colorless nail polish diluted in acetone; the exact thickness of the deposits was determined by trial and error so that a cover slip supported by the nail polish layers would hold lysed cells in position but still allow staining solutions to flow through at a practical rate. The built-up slide was warmed in a 37°C incubator, and a drop of lysis buffer (0.5% Triton X-100, 4 M glycerol, 100 mM MES KOH, 1 mM EGTA, pH 6.9) was placed between the nail polish stripes. A half cover slip bearing N115 cells was drained and carefully placed onto the drop of lysis buffer so that its ends were supported by nail polish. After 5 minutes at 37°C, excess buffer was removed by blotting with filter paper.

The method described above was successful in immobilizing the cytoskeletons of mitotic cells, but interfered with our usual method of fixation for immunofluorescent staining (a 5-min plunge into methanol at −20°C.) As an alternative way of preserving microtubules, cells were lysed in stabilizing conditions (4 M glycerol, 100 mM MES KOH, 1 mM EGTA, pH 6.9, 37°C) and the lysis buffer and staining solutions were 5 μM in the microtubule stabilizing drug, taxol(9) (a gift from Susan Horowitz, Albert Einstein College of Medicine, New York). After

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the initial lysis step at 37°C, microtubules remained intact while subsequent operations were carried out at room temperature.

Both microtubules and MTOC's were visualized by indirect immunofluorescent staining with tubulin antibody. The rabbit antiserum to tubulin was produced according to the Connolly et al. procedure (10) and purified by affinity chromatography on a column of immobilized tubulin by the method of Bustin et al. (11). Fluorescein conjugated goat anti-rabbit serum was purchased from Hyland Diagnostics Division, Travenol Laboratories, Inc., Costa Mesa, CA.

To apply staining solutions or rinses to immobilized cells, a 20-μl droplet was placed along one edge of the cover slip and replenished as necessary. Liquid was drawn under the cover slip by touching a piece of filter paper to the other side. Flow rates varied, depending on the gap between slide and cover slip, the density of cells on the cover slip, and the viscosity of solutions; rates of 10–60 s per 20-μl drop were suitable. The rate of flow of the lysis buffer and final mounting solution were slower because of their high viscosity.

After lysis, cells were rinsed with five droplets of phosphate buffered saline (PBS) (pH 7.3, containing 5 μM taxol) and then with three droplets of antitubulin diluted in PBS/taxol. After 20 min, two more droplets of primary antibody were applied and after an additional 40 min, the cells were rinsed with six droplets of PBS/taxol. Fluorescein conjugated secondary antibody was applied and rinsed in the same manner. After the final rinse, two droplets of PBS/taxol containing 10 μg/ml of the fluorescent, chromatin-staining dye bisbenzimid (Hoechst H33258) were flowed through (8), and the specimen was finished by flowing in mounting medium (PBS/50% glycerol/5 μM taxol/10 μg/ml bisbenzimid pH 7.8).

RESULTS

Interphase N115 nuclei are associated with groups of MTOC's as shown previously (4). A loose cluster of MTOC's in a single location near the nucleus is characteristic for interphase N115 cells (6, 7). We have also examined similar clusters of MTOC's by thin section electron microscopy and confirmed the results of Sharp, Osborn, and Weber (6) and Brinkley, et al. (3) that they are composed of single centrioles. The centrioles seem to display no preferred orientation or distance relative to each other, and no features distinguish one centriole from another. Centriole clusters similar to those of interphase cells were also observed at the poles of metaphase N115 cells by electron microscopy. Although immunofluorescence/thin section micrograph pairs were not obtained for the other stages of mitosis, there is no change in the immunofluorescent appearance or number of MTOC's at any point in the mitotic cycle, and we consider it likely that the MTOC's correspond at all times to singlet centrioles.

Seven cell-cycle stages can be distinguished in N115 cells by double fluorescent staining, with unique and characteristic arrangements of microtubules, MTOC's, and chromatin. We have interpreted these as interphase, prophase, early prometaphase, late prometaphase, metaphase, anaphase, and telophase. Since each stage can be clearly identified by the position and condition of the chromatin or chromosomes, it is possible to place the events affecting MTOC and microtubule arrangement into a well-defined sequence.

Intact cytoskeletons of interphase N115 cells are densely filled with microtubules, and it is generally difficult to view their MTOC's by tubulin immunofluorescence unless surrounding microtubules are eliminated. When microtubules are depolymerized by cold or drugs, or when interphase N115 nuclei are isolated, the MTOC's are seen as a cluster of bright bodies on one side of the nucleus (4). Using our preparation methods, the MTOC's of mitotic cells are seldom obscured by surrounding microtubules, but appear as bright dots at the centers of individual asters or grouped at the spindle poles. The ease in viewing MTOC's in mitotic cells does not appear to be due to any change in the size or brightness of the stained MTOC's themselves, but instead to a reorientation of microtubules, which now lie preponderantly on one side of each MTOC (facing the chromosomes).

An early prophase cell with five MTOC's and associated weak microtubule asters is shown in Fig. 1 *a*. At this stage, the MTOC's are somewhat spread apart compared to the usual interphase situation and microtubules are fewer, essentially confined to the asters immediately surrounding the MTOC's. For contrast, the cell at the lower left of Fig. 1 *a* is in interphase. Its microtubules are so abundant that individual fibers are lost and MTOC's are obscured in the overall bright staining. The same cells in bisbenzimid fluorescence are shown in Fig. 2 *b*. The interphase nucleus displays a characteristic bright and dark mottling due to differences in hetero- and euchromatin staining intensity. In the prophase nucleus, this mottling has taken on a finer, more granular appearance as chromosome condensation begins. The edge of the nucleus at this stage looks slightly serrated, presumably because the nuclear membrane is beginning to break down.

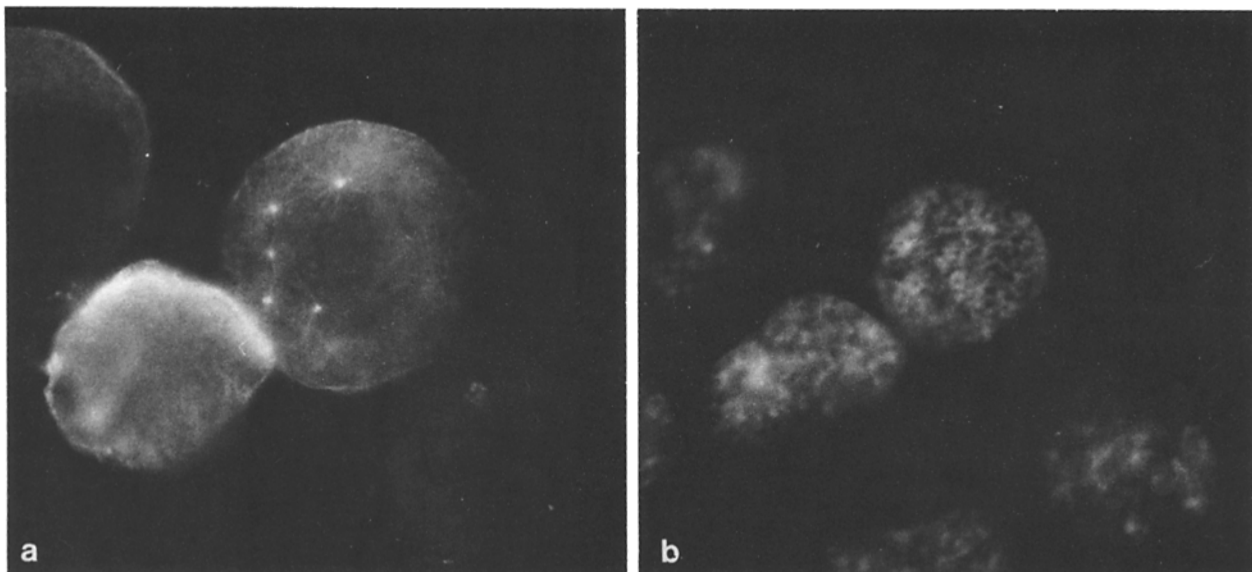

FIGURE 1 Prophase N115 cell. (*a*) Partially dispersed MTOC's with sparse microtubule asters; tubulin immunofluorescence. × 825. (*b*) Partially condensed chromatin; bisbenzimid fluorescence of the same cell.

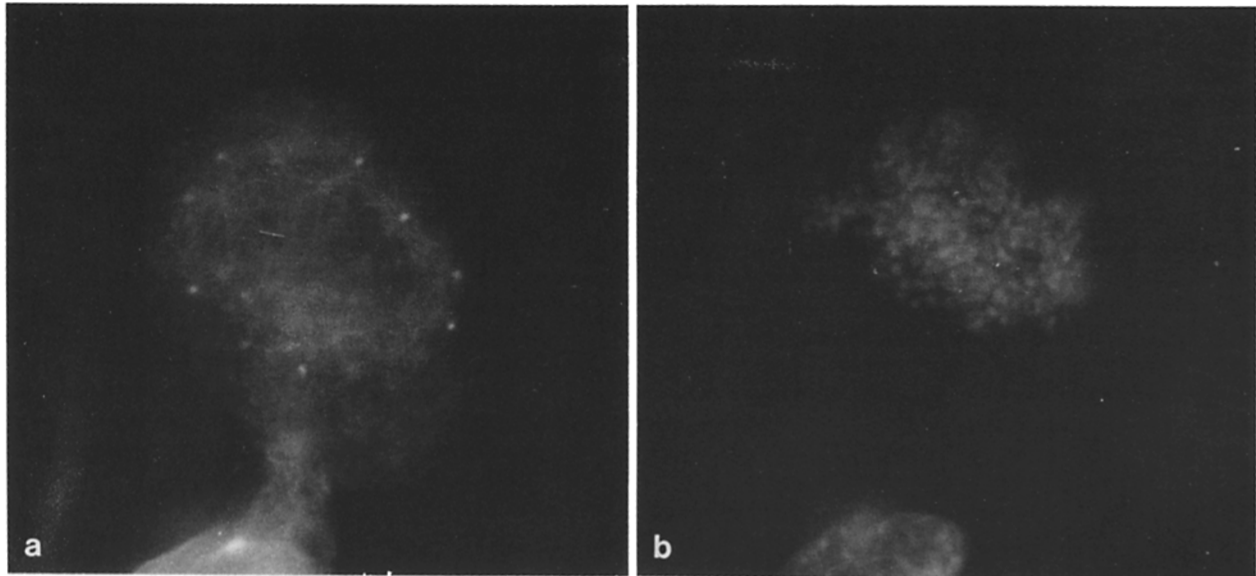

FIGURE 2 Early prometaphase N115 cell. (*a*) A plane of focus midway through the cell showing evenly dispersed MTOCs; tubulin immunofluorescence × 825. (*b*) Irregular mass of condensed chromosomes in the same cell; bisbenzimid fluorescence. × 825.

The dispersal of MTOC's which begins in early prophase continues, until at late prophase and early prometaphase the MTOC's are very evenly distributed around the former nuclear perimeter (Fig. 2*a*). Only a middle plane of focus is shown in the illustration; additional MTOC's were present above and below this plane, forming a complete ellipsoidal envelope in which individual MTOC's fit into an approximately geodesic arrangement. The bisbenzimid fluorescence picture of this cell (Fig. 2*b*) shows that the nucleus has been converted to a mass of condensed chromosomes, which is beginning to adopt a somewhat irregular shape.

In late prometaphase, the arrangement of MTOC's, as that of the chromatin, becomes less symmetric. Fig. 3*a* shows a late prometaphase cell with four microtubule asters, each growing from one or more MTOC's. Again, there were additional MTOC's and asters in other planes of focus. By late prometaphase, microtubule asters have become considerably denser than those seen during prophase or early prometaphase. At this stage, the corresponding DNA fluorescence image (Fig. 3*b*) shows an irregular mass of condensed chromosomes. Comparison of Fig. 3*a* and *c* with *b* and *d* reveals that the microtubule asters fit into pockets on the surface of the chromosome mass. Similar relationships have been seen with most prometaphase cells. Late prometaphase MTOC's occur singly, in small clusters, and frequently in lines or chains. A trough in the chromosome mass corresponds to such chains of MTOC's. We have not been able to subdivide late prometaphase into a series of distinct MTOC arrangements. Individual cells display a seemingly random selection of small clusters, chains and single MTOC's, and it is not possible to decide, for instance, whether clusters precede chains or vice versa.

Despite the apparent disorder of late prometaphase patterns, metaphase N115 cells show a consistent and simple arrangement. A side view of such a cell appears in Fig. 4*a*. The MTOC's are clustered at two poles (at least three can be seen at one pole and two at the other) and the microtubules form a conventional spindle between them. An edge-on view of chromosomes on the metaphase plate is seen in the corresponding bisbenzimid staining (Fig 6*b*). To better display the MTOC's at the mitotic poles, a view along the axis of the poles is shown in Fig. 5*a*. Fig. 5*a* shows the upper pole (with nine MTOC's) in tubulin immunofluorescence, the lower pole with six or seven MTOC's is not shown. The ringlike arrangement of the MTOC's is encountered quite frequently at metaphase and anaphase poles. Fig. 5*b* is a DNA fluorescence picture in a middle plane of focus, showing the chromosomes on the metaphase plate. The unequal chromosome distribution (dense at the edges of the plate, sparse or lacking at the center) is typical for N115 cells and has also been observed in other cell types (13).

The anaphase situation in N115 cells develops predictably from metaphase. MTOC's are still found at the mitotic poles (Fig. 6*a*) and again frequently form rings when seen end-on; Fig. 6*b* shows an anaphase cell in bisbenzimid staining. As anaphase progresses, the separating sets of chromosomes often become curved (in side view) with convex sides toward each other and concave sides facing the poles and clustered MTOC's.

During telophase, spindle microtubules degenerate into a midbody as expected, and MTOC's are found in fairly tight cluster on the opposite or outward sides of the daughter nuclei. In late telophase, new microtubules—presumably the beginning of the interphase network—are seen originating from the clustered MTOC's.

In all the studies we found a very low precentage of multipolar spindles or abnormal mitoses. In a typical case, two tripolar spindles were found out of four hundred cases. Although virtually all cells had two mitotic poles the number of MTOC's at each pole ranged from 2 to 13 and often unequal numbers were present at each pole.

DISCUSSION

As a preliminary to studying the behavior of N115 multiple MTOC's during mitosis, we wished to establish their physical nature, and in particular to determine whether the bright bodies observed at the foci of immunofluorescently stained microtubule asters correspond to a consistent ultrastructural component in thin sections of the same specimen. We obtained electron microscopic evidence, which agrees with other published studies (6, 7), indicating that all of the neuroblastoma

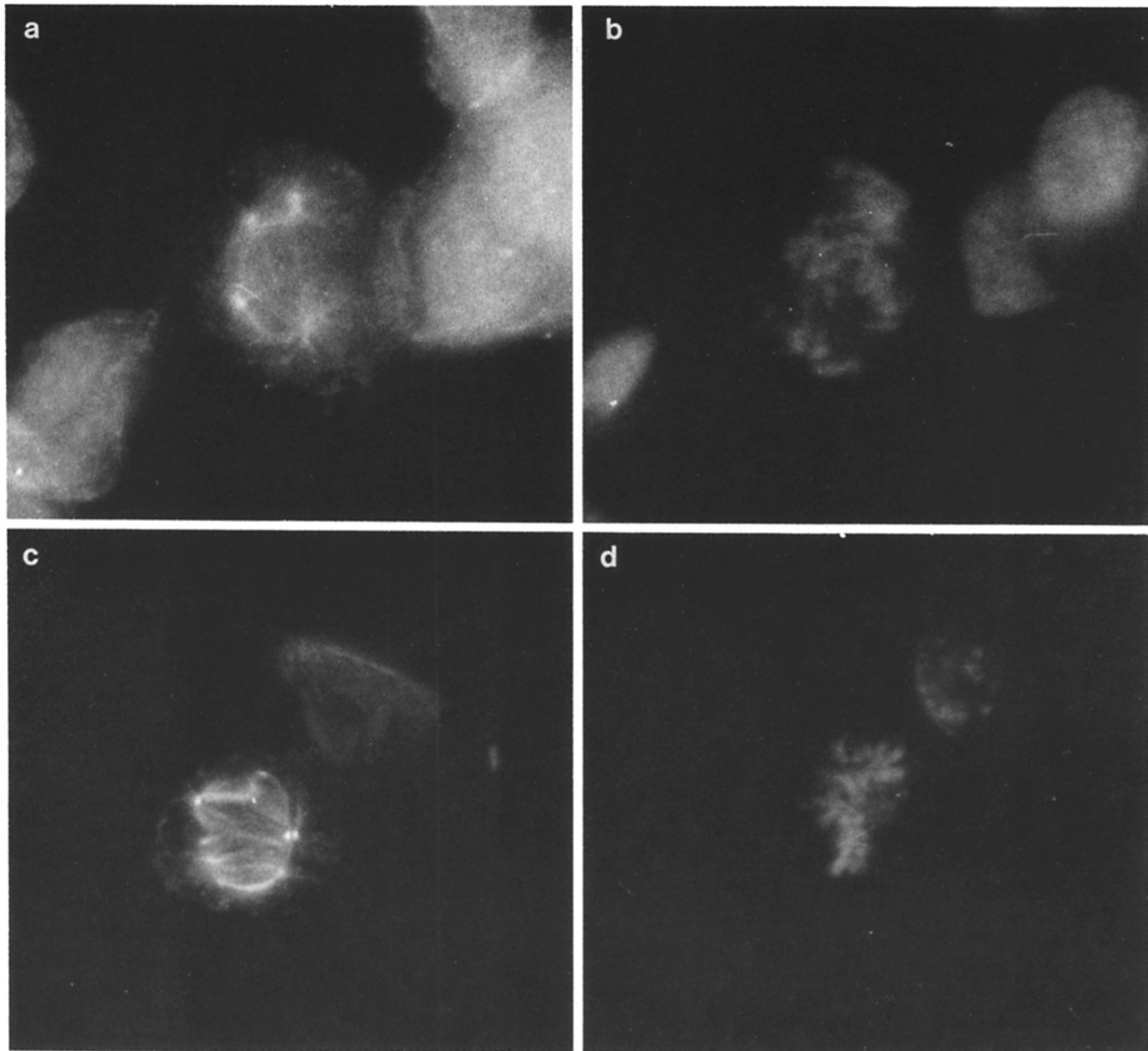

FIGURE 3 Late prometaphase N115 cell. (*a*) Tubulin immunofluorescence showing four microtubule asters. (*b*) Bisbenzimid fluorescence of the same cell showing condensed chromosome mass with pockets corresponding to positions of microtubule asters. (*c*) Tubulin immunofluorescence of another prometaphase N115 cell. (*d*) Bisbenzimid fluorescence showing a large trough in the chromosome mass corresponding to the MTOC positions. × 825.

MTOC's in interphase cells are centrioles. Similar results were seen in metaphase cells, where clusters of singlet centrioles were found at the mitotic poles. In no case did we find any other type of structure (e.g., the dense amorphous material which in some cases appears to nucleate microtubules in the absence of centrioles [14, 15]) corresponding to immunofluorescent MTOC's. Thus, we would favor the conclusion that all of the N115 MTOC's are centrioles, probably throughout the cell cycle. We also concur with the observation of Sharp, et al. (6) and Brinkley, et al. (7) that N115 centrioles are singlets, which are found in randomly oriented clusters rather than tightly associated right angle pairs. Immunofluorescent evidence, the intense staining of N115 MTOC's with antitubulin and with spontaneous antisera against centrioles (16), further supports the notion that neuroblastoma multiple MTOC's are centrioles. We have recently observed that N115 MTOC's in all phases of the cell cycle are labeled by two monoclonal antibodies (Ring, et al., [12]) that appear to recognize centrioles in several cell lines.

If a large percentage of the N115 MTOC's were not centrioles, it would perhaps be less surprising that, despite their numerous organizing centers, virtually all N115 cells undergo a normal bipolar mitosis. The fact that all N115 MTOC's are apparently identical singlet centioles makes this cell line an interesting test case for establishing the relative importance of the centriole versus other cellular structures in organizing the bipolarity of cell division. This is especially true because we have found that multiple and functional MTOC's are present at every stage of the cell cycle, and because the numbers of MTOC's at the two poles of dividing cells are frequently unequal.

Fig. 7 summarizes our observations on changes in the state and distribution of chromatin, microtubules, and MTOC's during the division of N115 cells. The most noticeable events affecting MTOC distribution begin with a dispersal during late interphase or early prophase, which leads to a very evenly spaced arrangement of MTOC's around the former nucleus during late prophase and early prometaphase. As prometa-

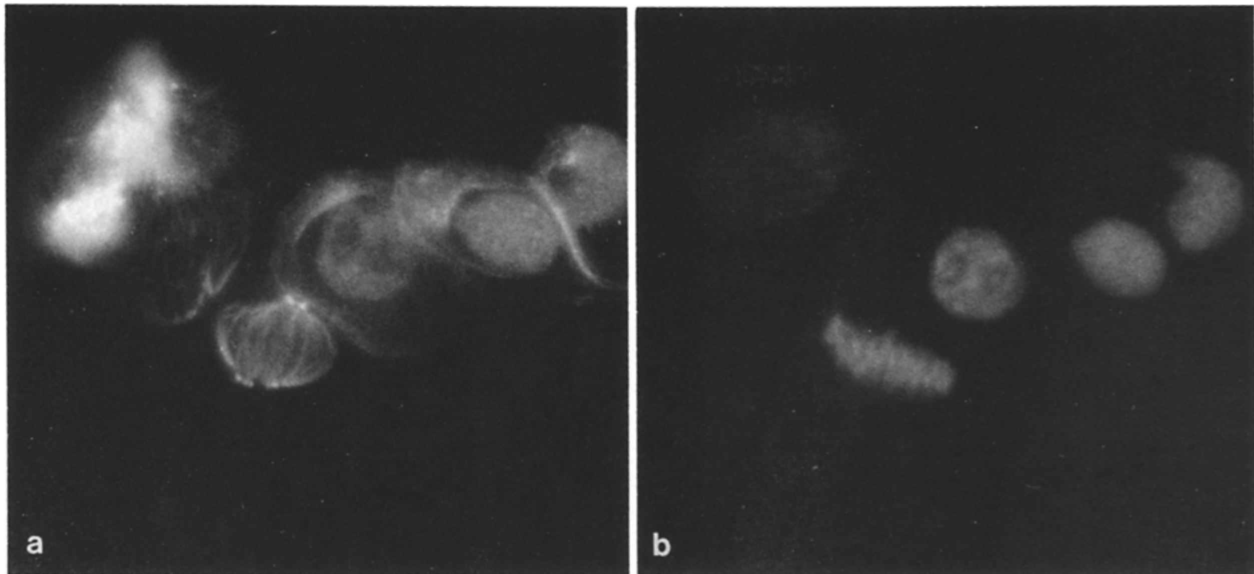

FIGURE 4 Side view of metaphase N115 cell. (*a*) Tubulin immunofluorescence showing metaphase micotubule spindle with MTOCs at each pole. (*b*) Bisbenzimid fluorescence of the same cell showing chromosomes in metaphase plate. × 825.

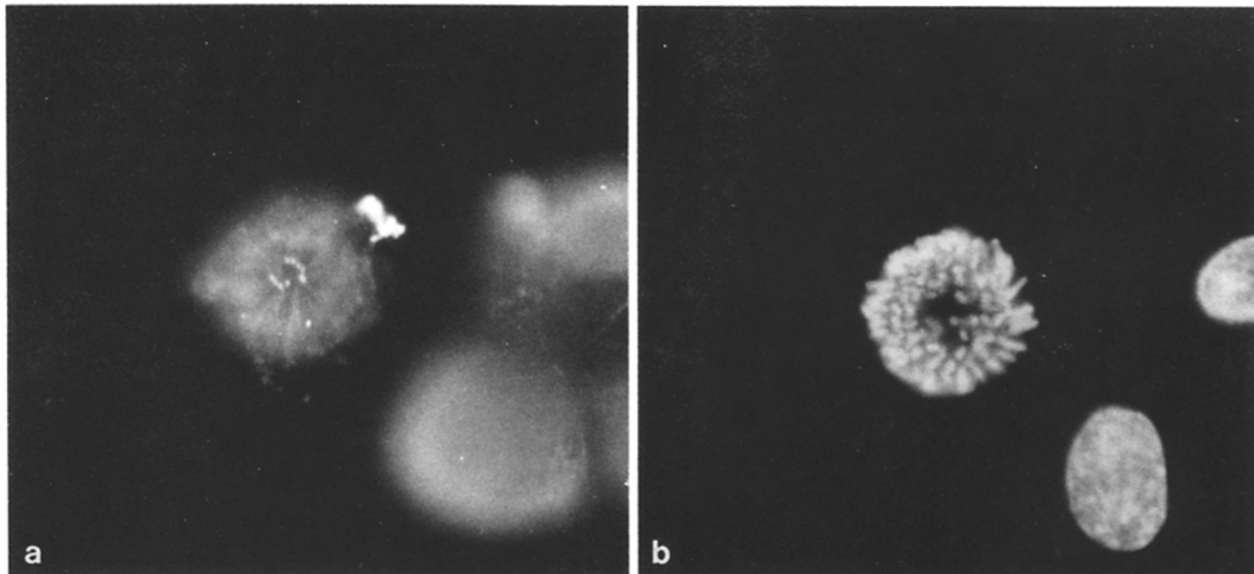

FIGURE 5 End view of metaphase N115 cell. (*a*) Upper pole in tubulin immunofluorescence showing ring of nine MTOCs. (*b*) Metaphase plate in bisbenzimid staining of the same cell showing chromosomes.

phase continues, the MTOC's form irregular clusters and chains, which gradually simplify until all MTOC's are confined to two clusters (often ringlike) at the metaphase poles. Subsequently, MTOC's remain at the poles during anaphase and telophase, at which time any ringlike arrangements revert to irregular clusters.

These observations answer a number of the questions that we posed initially. Multiple MTOC's do persist and function in microtubule organization throughout the N115 cell cycle. Furthermore, the MTOC's go through a series of distinct and consistent rearrangements, which can be assigned to definite stages of mitosis on the basis of chromatin and microtubule distribution. Two questions in particular require further consideration. First, what is the mechanism or mechanisms responsible for the dispersal of MTOC's at prophase and the aggregation of MTOC's at the mitotic poles after prometaphase? Second, what mechanism ensures formation of a bipolar spindle in the presence of so many apparently identical centrioles?

Two sorts of explanations can be conceived for the prophase dispersal of MTOC's. One alternative is that MTOC's are moved into their new positions by interaction with components of the cytoskeleton that are not visualized by our staining procedures (e.g., intermediate filaments, microfilaments, nuclear envelope). A second possibility is that MTOC movement specifically depends on microtubule extension or interaction of microtubules with surrounding structures. Compared with other stages of the N115 cell cycle, we observed the least microtubules during the early prophase MTOC dispersal. A similar scarcity of microtubules at prophase has been noticed in many cell types. The prophase microtubules that we observe appear as sparse, fairly symmetrical asters about the MTOC's

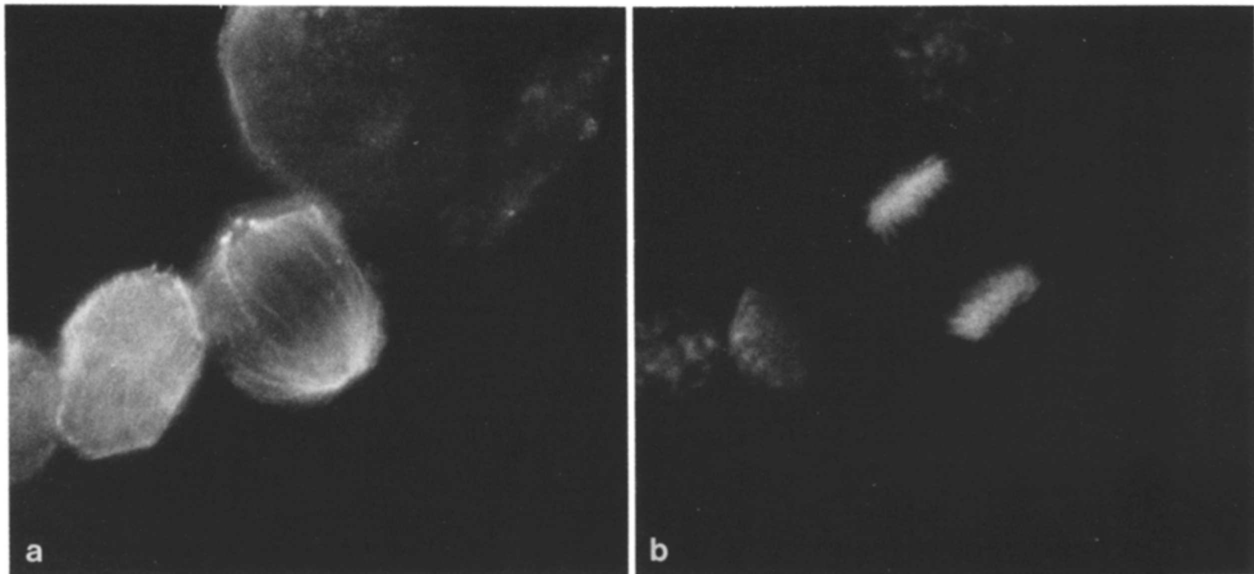

FIGURE 6 Anaphase N115 cell. (*a*) Tubulin immunofluorescence showing anaphase microtubule spindle and the MTOCs clustered at one pole. (*b*) Bisbenzimid fluorescence of the same cell showing the separating chromosomes.

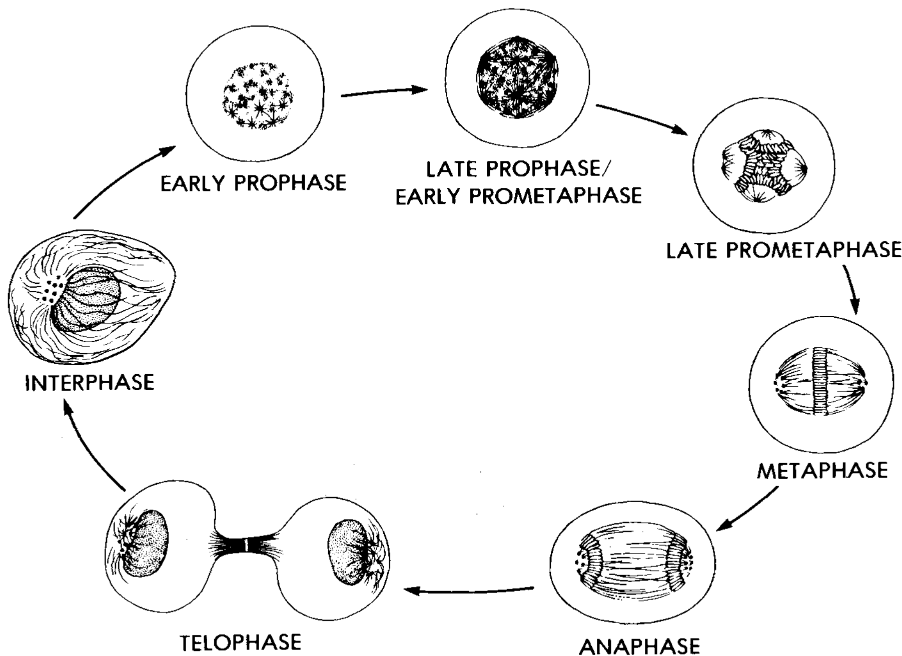

FIGURE 7 Composite drawing illustrating the rearrangements of MTOCs, microtubules, and chromatin during the N115 cell cycle.

and closely resemble the beginning stages of microtubule regrowth after drug or cold treatment. Each prophase MTOC and aster appears independent—i.e., there is no observable tendency for the separating MTOC's to form pairs connected by spindlelike microtubule arrangements. Instead, the microtubules of neighboring asters often end short of contact. Any motive force related to microtubule extension, then, would seem to depend on interaction of the growing asters with nonmicrotubule components.

It is possible that the scarcity of prophase microtubule in our experiments occurs because a cell-cycle-dependent change in composition renders these microtubules more susceptible to breakdown under our preparation conditions. However, if the situation we observe is real, as suggested by other observations of reduced microtubule numbers at prophase, then the resemblance of our prophase asters to those seen in microtubule regrowth experiments may indicate that microtubules in the cell have broken down just before prophase. Such breakdown may in fact be necessary, since the extensive interphase microtubule network could easily be imagined to interfere with rearrangement of the MTOC's.

It is possible that microtubules are detached from the MTOC's leading to transient depolymerization of the released microtubules (17). This in turn would raise the level of available tubulin monomers and stimulate the extension of newly nucleated microtubules from the MTOC's. Such a phenomenon would explain the loss of the numerous interphase microtubules and the appearance of the sparse microtubule asters resembling regrowth in our prophase cells. The release of astral fibers from the mitotic center has been observed for many years and

successive shedding of the fiber has been termed "moulting" by Wilson (18) (also P. Harris, personal communication). The dispersal of MTOC's may then be simultaneous with and possibly dependent on extension of new microtubules. We have not observed any prophase cells that completely lack microtubules, but this may only indicate that any period of total microtubule breakdown at the beginning of prophase is very brief.

A second rearrangement occurs during the latter part of prometaphase as MTOC's aggregate at the spindle poles. Consideration of the processes involved in this event is closely tied to the remaining question of how N115 cells guarantee formation of a bipolar spindle. In most cells, it is easy to view the duplicated centriole as a preexisting source of bipolarity. Perhaps the simplest explanation of the situation in N115 cells is to assume that, despite their multiple centrioles, these cells also contain a single master organizing structure, which is duplicated before mitosis. The master organizer could be either a specialized centriole—functionally distinct but indistinguishable by immunofluorescence or electron microscopy—or it could be another cytoskeletal structure not visualized by our techniques. In either case, the metaphase clustering of MTOC's would result from their interaction with the two copies of the duplicated master organizing structure.

Another source of bipolarity in prometaphase cells may be the duplicated chromosomes themselves. The separating chromatids of a metaphase chromosome bear exposed kinetochores facing in opposite directions. During prophase or prometaphase, it seems likely that only one of the opposed kinetochores on each chromatid pair can form a connection via microtubules to any given MTOC. With the further assumption that some mechanism, perhaps involving chromatin interactions between neighboring chromosomes, assembles the chromosomes into a metaphase plate with all kinetochores facing outward from the plate surfaces, it is then possible to visualize how MTOC's, having already formed connections to certain kinetochores, would be oriented opposite to one or the other plate surface. In other words, a bipolar spindle may be the result rather than the cause of metaphase chromosome positioning.

Following this speculative model, the observed correspondence between early prometaphase microtubule asters and pockets in the chromosome mass would represent the establishment of MTOC-kinetochore linkages. The irregular clusters and chains of MTOC's seen in later prometaphase would be intermediate stages occurring as individual pockets merged and the chromosome configuration tended toward the metaphase plate. At the boundary of two pockets, continued microtubule growth could force chromosomes to penetrate the edge of the pocket and expose their free kinetochore to the MTOC of a neighboring pocket. On the other hand, if several MTOC's formed initial attachments to the kinetochores on one side of a group of chromosomes, their individual pockets would tend to merge. With allowance for breakage of a certain number of conflicting attachments, the processes of merging and edge formation could continue until the metaphase plate is formed. Whether a particular MTOC ended at one mitotic pole or the other would depend on the metaphase orientation of its corresponding kinetochores. If the initial selection of kinetochores is random, there would be no driving force to provide equal numbers of MTOC's at the poles, explaining our observation that these numbers can be highly disparate.

Some early observations on mitosis in the parasitic wasp *Achroschismus* may be relevant to the idea presented above, that kinetochores could determine spindle bipolarity. Schrader noted that during prophase, each of the chromosomes in this organism develops a clear spindle-shaped zone around itself (19). Although the techniques available at the time were not able to visualize individual microtubules or centrioles, the fusiform shape of the individual spindles suggests that their fibers were anchored to some sort of organizing center at each pole. From an originally random orientation, the individual spindles gradually became parallel and coalesced into a single large spindle. The fact that each chromosome in *Achroschismus* formed a complete miniature bipolar spindle suggests even more strongly for *Achroschismus* than N115 cells that spindle formation may sometimes be dominated by kinetochores.

After the metaphase configuration is established in N115 cells, the continuation through anaphase and telophase involves no unexpected rearrangements of MTOC's. The clustered MTOC's remain at the poles, and therefore precede the separating chromosomes and daughter nuclei during cytokinesis. The same ordering, with centrioles in front of nuclei, has been observed in various types of migrating interphase cells (20).

In summary, our experiments with mitotic N115 cells support the conclusions of Spiegelman, et al. (4) that N115 cells contain multiple MTOC's, and that these MTOC's move in specific patterns associated with general transformations of the cell (in the former case, neurite outgrowth; in the present case, cell division). In agreement with previous observations (6, 7), we find that each N115 MTOC is a singlet centriole. The multiple N115 MTOC's persist and maintain asters of microtubules throughout the entire cell cycle; nevertheless, N115 cells exhibit a normal bipolar mitosis. A striking observation is that the MTOC's or centrioles are often allocated unequally to the two poles. These results have led us to question whether the duality of the mitotic spindle depends on duality of functional centrioles. As an alternative hypothesis, we suggest that the multiple centrioles of N115 cells become associated with the paired kinetochores of duplicated chromosomes, and that subsequent formation of the metaphase plate may be the cause, rather than the effect, of bipolar spindle formation in this cell type.

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