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Microtubule Distribution in Unequal Cell Division of the Grasshopper Neuroblasts

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Introduction

The fibrous structure of the mitotic apparatus is mainly composed of microtubules formed by association of tubulin molecules. Microtubule arrangement within the cell can be demonstrated by immunofluorescence microscopy with anti-tubulin antibody (Weber and Osborn 1979, Brinkley *et al.* 1980). Furthermore, a wide range of species cross-reactivity of tubulin antibodies, which is attributed to the conservative nature of tubulins (Dales 1972), has made tubulin immunofluorescence a useful approach for the study of microtubules.

Neuroblasts of grasshopper embryos divide unequally to form a large daughter neuroblast and a small daughter ganglion cell. Since the cleavage plane is closely related to the position of mitotic apparatus, the behavior of the mitotic apparatus is different between in unequal cytokinesis and in equal cytokinesis. Unequal cytokinesis has been described in detail in spiralia eggs and sea urchin eggs, in which the eccentric location and asymmetrical configuration of the mitotic apparatus are commonly observed (Dan 1978 1979, Dan *et al.* 1983, Dan and Ito 1984, Schroeder 1987). In the grasshopper neuroblasts, the mitotic apparatus shifts from the cell center to an eccentric position during middle anaphase, but asymmetrical asters have not been clearly demonstrated. In the present study, the changes in the microtubule pattern during mitosis, as well as interphase, are traced by immunofluorescence microscopy.

Materials and Methods

Preparation of grasshopper embryos

Embryos of *Chortophaga viridifasciata* (De Geer), 15~16 days old, which had been kept at 26°C were used throughout the present study. Embryos were removed from the egg in Carlson's medium (Carlson and Gaulden 1964).

Antibodies

Polyclonal anti-tubulin antibody against purified tubulin from bovine brain raised in a rabbit was generously provided by Dr. Yoshida (1983). Monoclonal antibody YOL1/34, a rat antibody against yeast tubulin (Serotec Ltd., England), which is reputed to bind specifically to the alpha subunit of tubulin (Kilmartin *et al.* 1982), was also used. FITC-conjugated goat anti-rabbit IgG was purchased from MBL Co Ltd. (Japan), and FITC-conjugated goat anti-rat IgG was obtained from Cappel Laboratories Inc. (USA).

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SDS-polyacrylamide gel electrophoresis and immunoblot staining

Total *Chortophaga* proteins were extracted from embroys by lysing with 2% Nonidet P-40, 0.5 mM phenylmethylsulfonylfluoride and 1 mM EDTA in 10 mM Tris-buffered saline (TBS, pH 7.4).

Electrophoresis on SDS-polyacrylamide gel was performed by the method of Laemmli (1970). After the gel electrophoresis, protein bands were transformed to Durapore membrane (Millipore Ltd., Japan) electrophoretically as described by Towbin *et al.* (1979), and the membrane was stained by avidin-biotin complex methods with anti-tubulin antibody (10 μg/ml).

Indirect immunofluorescence

The head and thoracic segments of embryo, in which many neuroblasts are contained, were placed on a poly-L-lysine coated coverslip (Mazia *et al.* 1975) in a small amount of Carlson's medium. The neuroblasts were quickly dissociated from the segments by using a pair of glassneedles, and then the coverslip was immediately immersed in a fixative. The fixative was made of 4% paraformaldehyde mixed in PEM buffer (100 mM PIPES, 2 mM EGTA, 1 mM $MgCl_2 \cdot 6H_2O$, 0.1 M NaCl, adjusted to pH 7.0 with HCl) and 1% saponin for permeabilization. The specimens were fixed for 30～60 min at room temperature, washed with PEM buffer, and treated by methanol for 10 min at 4°C. The coverslip was air-dried, and immediately immersed in 10 mM TBS (pH 7.6). The cells on the coverslip were incubated with the anti-tubulin antibody overnight in a humid condition at room temperature. After repeated washes in TBS, the cells were incubated with FITC-labeled second antibody for 2 hr at room temperature. The coverslip was washed thoroughly in TBS and mounted on a slide with 70% glycerin in TBS (pH 9.0) containing 5% n-propyl gallate (Giloh and Sedat 1982).

In control studies for tubulin-specific immunofluorescence, the cells were incubated with only the second antibody.

The stained cells were examined with a Nikon fluorescence microscope equipped with Nikon B21 filter. Photographs were taken with a Nikon CF UV-F 100/1.3 objective lens. Fluorescent images were recorded on Kodak Tri-X films.

The mitotic stages shown in the present study are classified in accordance with Carlson (1946).

Colcemid treatment

The neuroblasts in the head and the thoracic segments were incubated with 5 μg/ml colcemid in Carlson's medium for 2 hr. The treated cells were prepared for immunofluorescence microscopy as described above.

Results

Characterization of Two Anti-tubulin Antibodies

In this study, the arrays of microtubules in grasshopper neuroblasts were demonstrated by indirect immunofluorescent staining. Since the antibodies used were previously prepared against mammalian and yeast antigens, immunoblot analyses were performed to determine their reactivity against the whole grasshopper

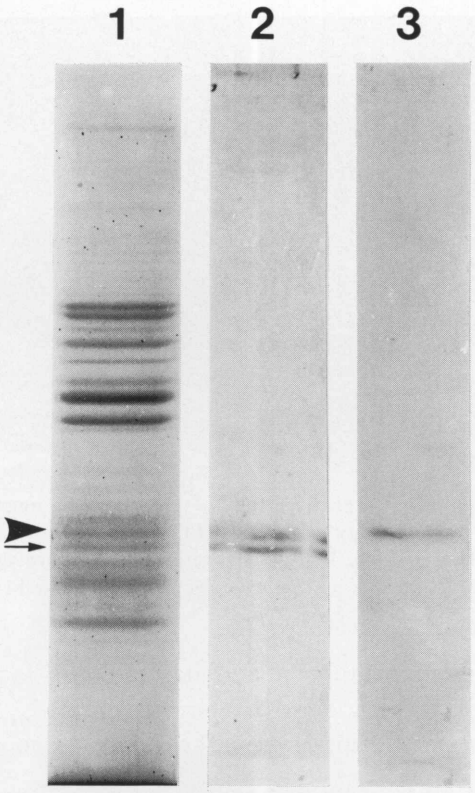


Fig. 1. Characterization of anti-tubulin antibody by immunoblot staining. Electrophoresis of the neuroblasts extract was performed on 7.5% polyacrylamide gels. The proteins on the gel were stained with Coomassie brilliant blue (lane 1). The proteins transferred to the membrane were stained avidin-biotin complex methods with affinity-purified anti-bovine tubulin rabbit antibody (lane 2), or monoclonal anti-yeast tubulin rat antibody (lane 3). Arrowhead and arrow indicate α-, β-tubulin respectively.

embryo extracts. As shown in Fig. 1, the two antibodies used in this study reacted only with tubulin bands on immunoblot staining of grasshopper embryo proteins; the monoclonal antibody reacted with alpha tubulin and the polyclonal antibody reacted with both alpha and beta tubulins. These facts indicate that the antibodies are monospecific for tubulin in grasshopper extracts.

Microtubule Distribution in Normal Neuroblasts

The neuroblasts are in a contact with cap cells in one of the polar surface, and produce columns of ganglion cells opposite to the cap cells. In all figures, the cells are oriented with the side to which the cap cells are attached (hereinafter referred to as the cap cell side, cc-side) at the top of the figure and the side from which the ganglion cell columns extends (hereinafter referred to as the ganglion cell side, gc-side) at the bottom.

A similar fluorescence pattern of microtubules was revealed in both cases of

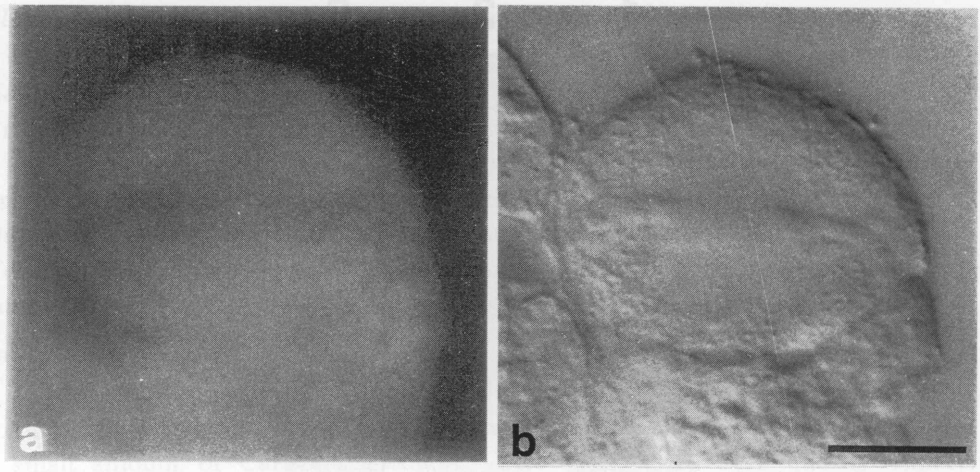


Fig. 2. Indirect immunofluorescence pattern of a metaphase neuroblast stained with the second antibody only (a). The chromosome shown as dark spots are arranged on the equator, but no fluorescent fibers are detected. Very faint fluorescence is observed in the cytoplasm. (b) Nomarski interference micrograph of the same cell. Bar, 10 μm.

staining with the polyclonal anti-tubulin antibody and the monoclonal one.

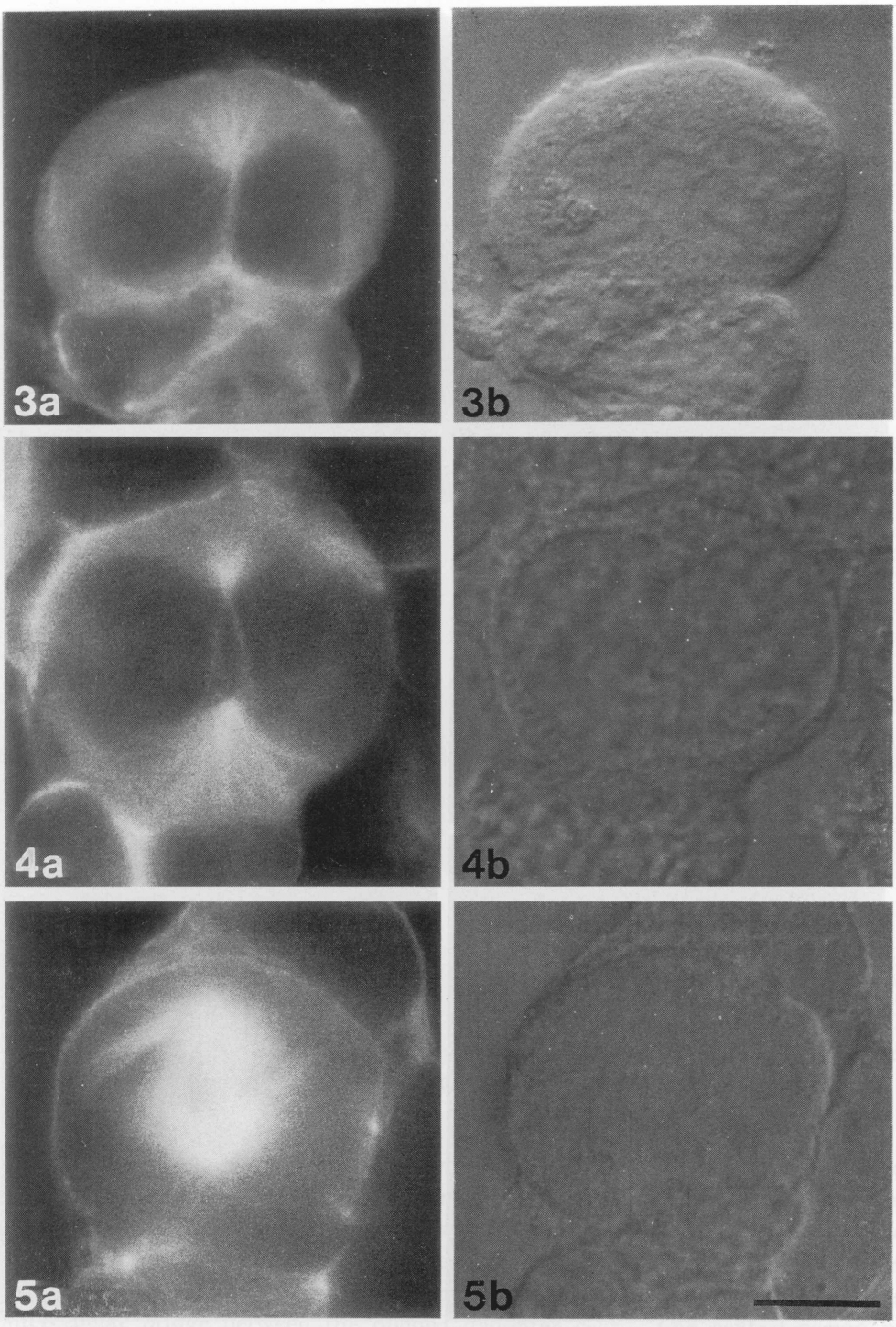
In control cells stained with only the second antibody, no fluorescent fibers were detected, although very faint nonspecific fluorescence was observed in the cytoplasm (Fig. 2).

late prophase ~ metaphase

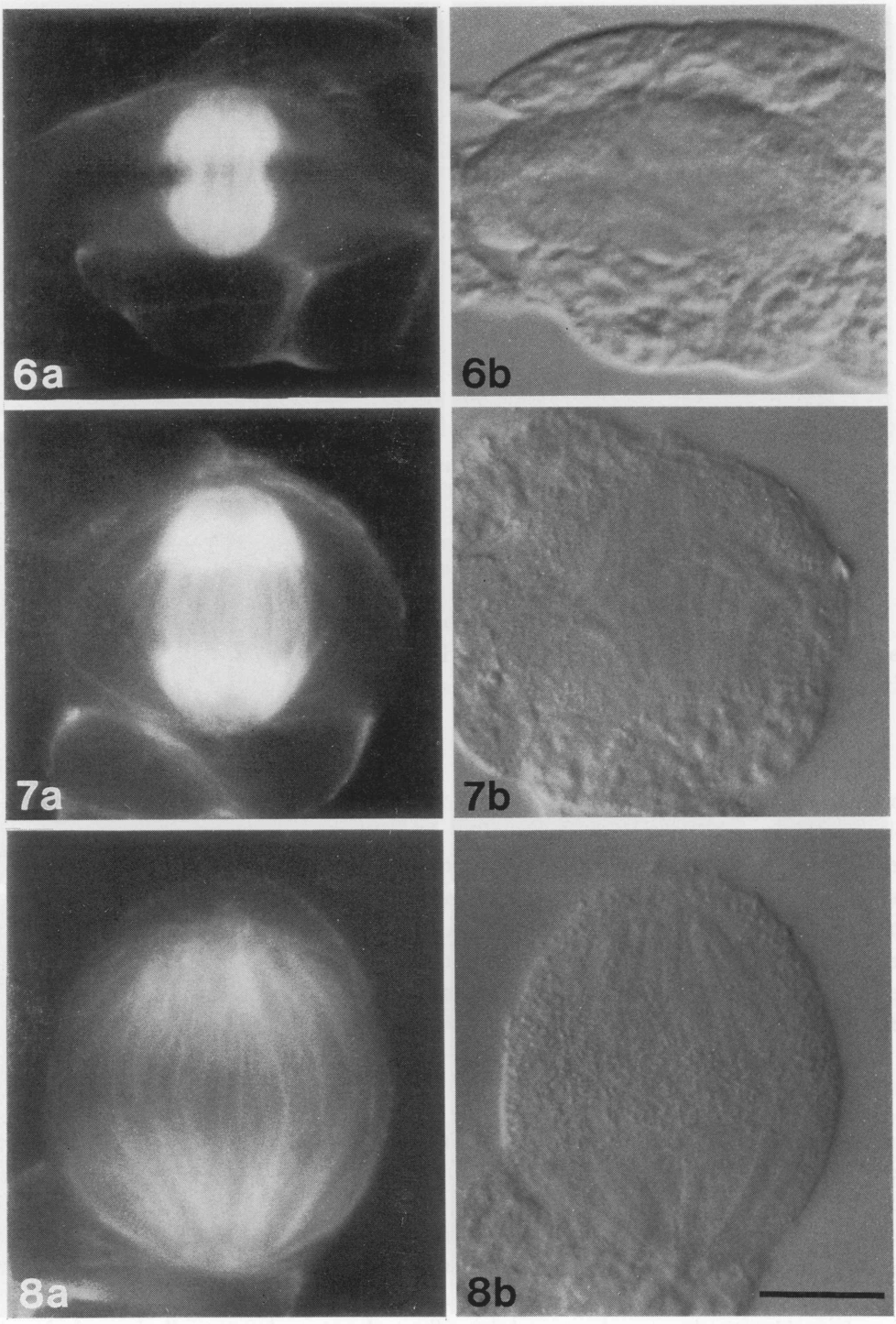
The fluorescent mitotic apparatus first appeared at late prophase when chromosomes were well-formed within the nucleus (Fig. 3). Astral rays extended radially from two poles, whereas continuous spindle fibers in the nuclear core were seen with careful focusing. When the cell assumed a spherical shape at very late prophase, well-developed asters of equal size were radiated from two bright spots which were connected by continuous spindle fibers (Fig. 4). After the nuclear membrane broke down, spindle formation progressed rapidly. Astral rays were extended most at prometaphase (Fig. 5), whereas they became very short at metaphase (Fig. 6).

anaphase

At early anaphase, a part of astral rays extended along the cell contour toward the cell equator, although most of astral rays were still short as in metaphase cells (Fig. 7). As the daughter chromosomes separated, the fluorescence of the spindle was reduced, especially in the interzonal region. At middle anaphase, the cell assumed an ellipsoidal shape and the spindle elongated remarkably. At the same time, the spindle began to shift from the cell center to the gc-side. The gc-side pole of the spindle was close to the cell periphery, whereas the cc-side pole was apart from the cell cortex, as shown in Fig. 8. The size of asters at the both poles appeared to be equal. Some astral rays which directed toward the polar cortex remained short even in the cc-side (Fig. 8). At the onset of cleavage, astral



Figures 3–5. Fluorescent micrograph (a) and Nomarski interference micrograph (b) of the same cell after treatment with anti-tubulin antibody. Fig. 3, late prophase. Pole of gc-side is out of focus. Fig. 4, very late prophase. Fig. 5, prometaphase. Bar, 10 μm.



Figures 6–8. Fluorescent micrograph (a) and Nomarski interference micrograph (b) of the same cell after treatment with anti-tubulin antibody. Fig. 6, metaphase. Fig. 7, early anaphase. Fig. 8, middle anaphase. Bar, 10 μm.

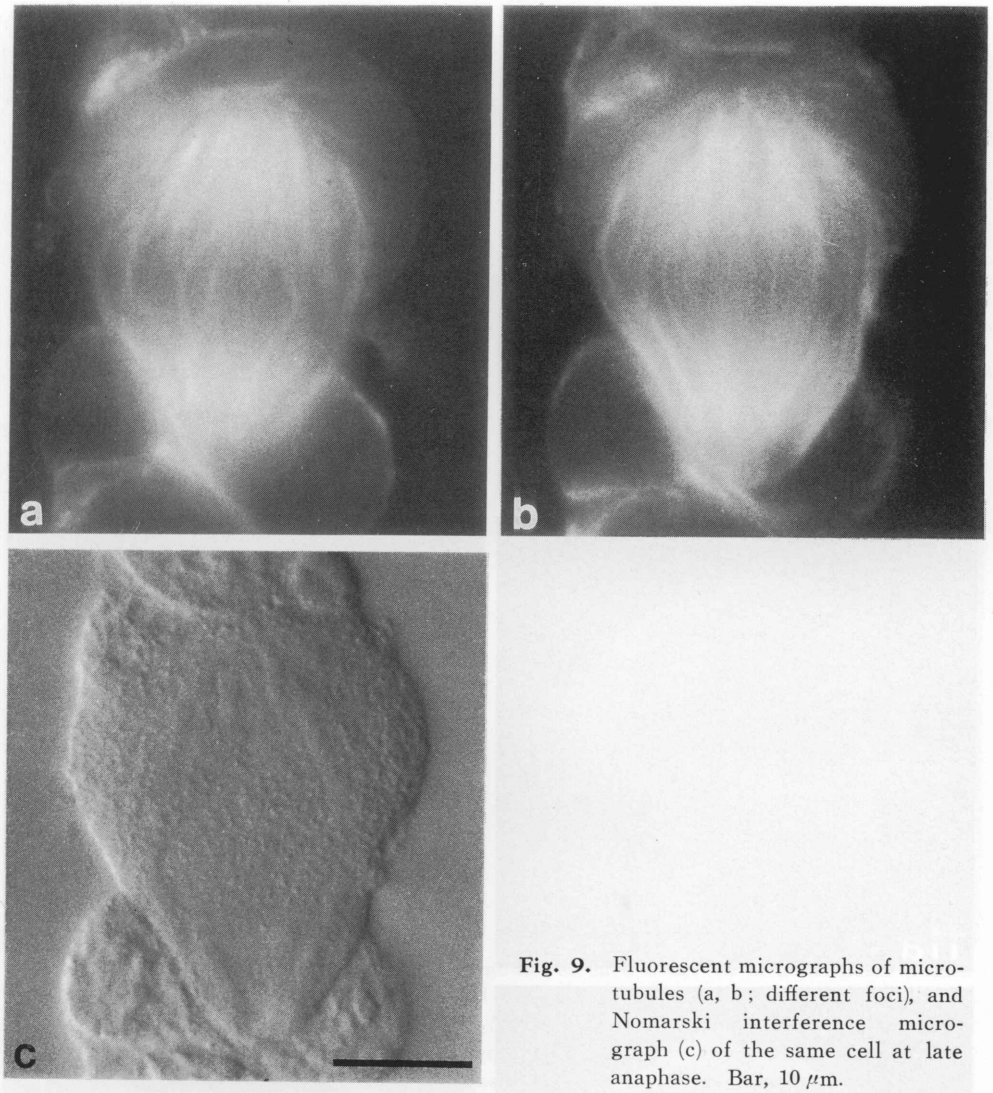
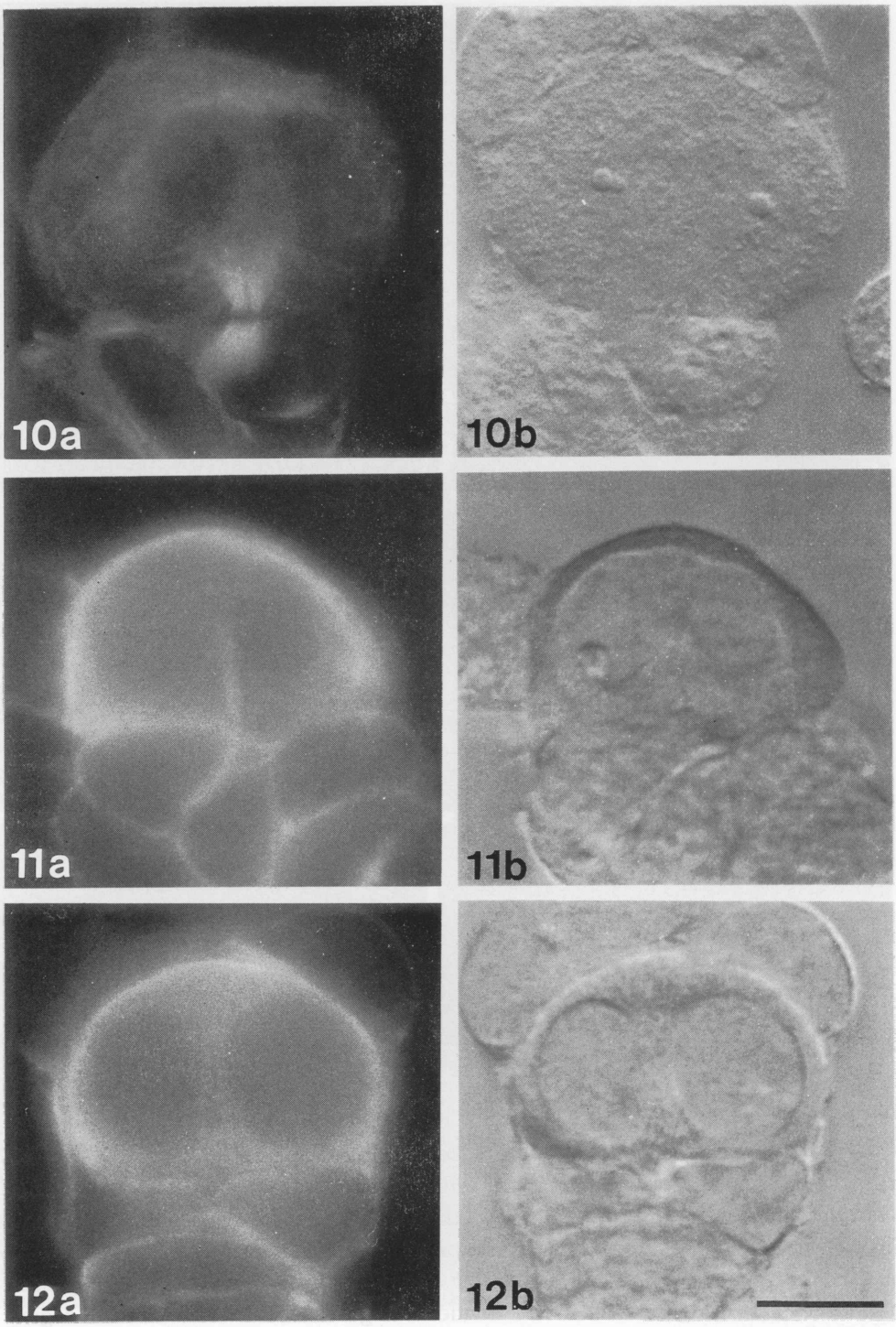


Fig. 9. Fluorescent micrographs of microtubules (a, b; different foci), and Nomarski interference micrograph (c) of the same cell at late anaphase. Bar, 10 μm.

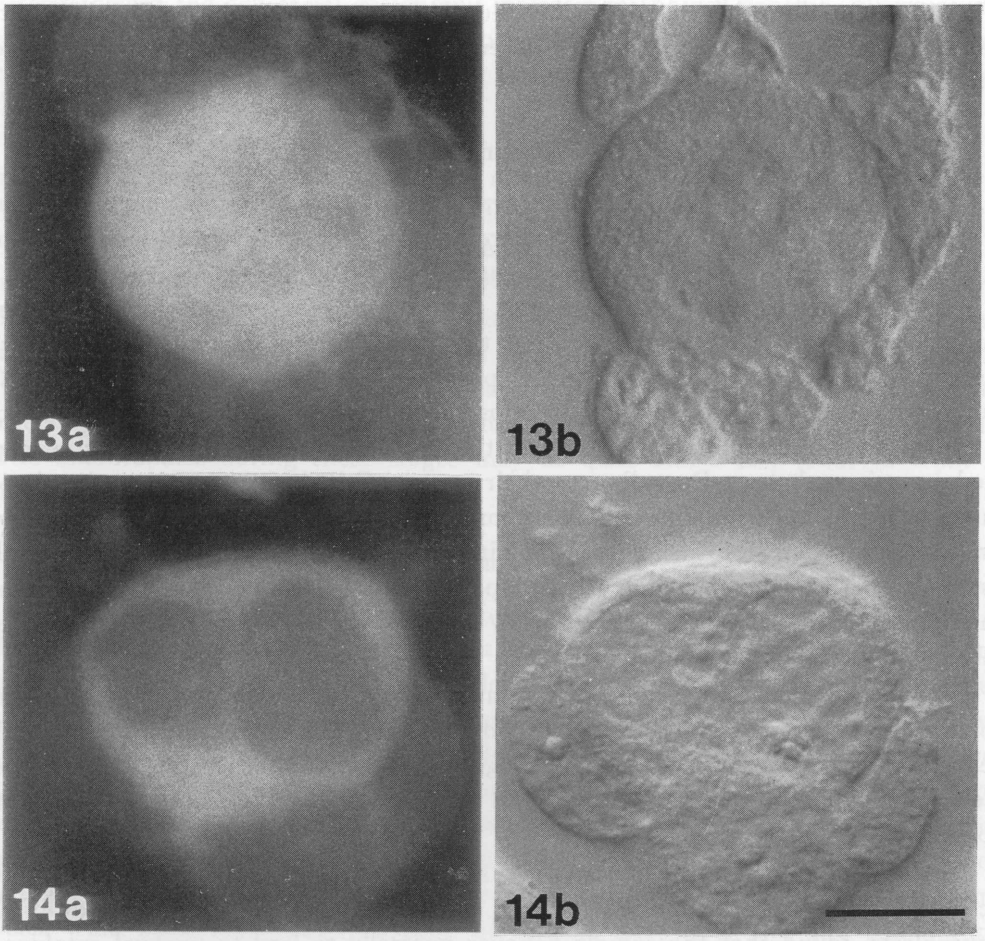
rays in the cc-side extended, while the aster in the gc-side could hardly be detected in the narrow space between the spindle and the cell periphery (Fig. 9).

telophase

A doughnut-shaped nucleus with a central cytoplasmic core was formed in the large daughter neuroblast, whereas an ordinary shaped nucleus was produced in the small daughter ganglion cell. In the daughter neuroblasts at middle telophase (Fig. 10), weak fluorescent fibers were present around the nucleus. Microtubular spindle remnants were bunched together by the cleavage furrow, and appeared as an intercellular bridge between the divided daughter cells. The central region of the fiber bundle, the mid-body, was not stained with anti-tubulin. In the daughter ganglion cells no fluorescent fibers other than the spindle remnant were seen. The fluorescent intercellular bridge became very thin at late telophase (Fig. 11), and



Figures 10–12. Fluorescent micrograph (a) and Nomarski interference micrograph (b) of the same cell after staining of anti-tubulin antibody. Fig. 10, middle telophase. Fig. 11, late telophase. Fig. 12, interphase. Bar, 10 μm.



Figures 13 and 14. Indirect tubulin immunofluorescence observed in neuroblasts treated with 5 μg/ml colcemid for 2 hr (a). Nomarski interference micrograph of the same cell (b). Fig. 13, "star" metaphase which is arrested in mitosis with colcemid. No mitotic microtubules are detected. Fig. 14, middle prophase. Fluorescent fibers remain in the nuclear core even after colcemid treatment. Bar, 10 μm.

disappeared by the time the cells entered interphase.

interphase ~ middle prophase

The doughnut-shaped nucleus occupied a larger part of the hemispherical cell at interphase. Fluorescent fibrous structures were seen only in the nuclear core (Fig. 12). These fibers ran parallel to the nuclear core. No fluorescent spot to which some fibers converged was observed. During early and middle prophase, microtubule pattern was the same as in interphase cells.

Effects of Colcemid

When neuroblasts were treated by 5 μg/ml colcemid for 2 hr, spindle formation was completely inhibited. The chromosomes scattered in all directions to form a "star" metaphase. No fluorescent fibers were detected in the "star" metaphase

(Fig. 13). On the other hand, in the neuroblasts from interphase till middle prophase a few fluorescent fibers were observed in the nuclear core cytoplasm even after colcemid treatment (Fig. 14).

Discussion

In the animal cell division, the cleavage furrow forms across the middle part of the spindle without exception. Therefore, unequal cytokinesis involves some mechanism which gives rise to the eccentric location of the spindle in the cell.

In unequal cytokinesis of the fourth cleavage of sea urchin eggs, the resting nucleus moves to the vegetal pole, and the newly formed mitotic apparatus was already found to be located in eccentric position (Dan 1978 1979, Dan *et al.* 1983). Lutz and Inoué (1982) found that the movement of the nucleus to the vegetal pole is prevented by colcemid treatment, and that the migration of the nucleus is mediated by microtubules. Preceding to their report, Czihak (1973) proposed a hypothesis that the nuclear migration is accomplished by the differential growth of two asters. Schroeder (1987) showed by indirect tubulin immunofluorescence that the asters are already formed and oriented parallel to the future division axis around the time of nucleus migration, but they are not too extensive to cause nuclear migration, as proposed by Czihak.

In unequal cleavage of *Spisula*, Dan and Ito (1984) observed that the metaphase spindle moves to one side of the cell, and the aster which makes contact with the cell cortex becomes flattened. They judged, however, that the size difference between the two asters is not so great as to account for the shift of the mitotic apparatus.

In the grasshopper neuroblasts, the spindle shifts from the cell center to the gc-side during middle anaphase. Carlson (1952), in his microdissection study of the neuroblasts, found that the spindle behaves normally to lead to unequal cytokinesis after it is rotated approximately 180° at early anaphase. Kawamura (1977) reported that normal cytokinesis occurs when 180° rotation is done at middle anaphase. The present study shows that the size of the two asters at the spindle poles are equal up to the middle of the spindle shifting, becoming different after the completion of the spindle shifting. While the astral size between the two poles is not different before middle anaphase, reversing the poles by microdissection may not disturb the normal shifting of spindle to the gc-side. From the present observations, it is considered that the spindle shifting is not caused by the differential growth of asters at the two poles, but the difference in the configuration of the two asters may possibly be induced by some environmental conditions after completion of the spindle shifting.

Kawamura (1977), in his microdissection study of the grasshopper neuroblasts, obtained results suggesting that the differential cortical movements, namely expansion at cc-side and shrinkage at gc-side, alters the relative position of the spindle toward the gc-side. In observation of the living neuroblasts, spindles never show movement to the cc-side, while the cytoplasm on the gc-side is flowing into the cc-side. The present observation reveals that, during early and middle anaphase, the astral rays

extend toward the equatorial cortex and intersect in the cell equator. It is assumed that these astral rays moor the spindle to the equatorial cortex during the differential cortical movements, so that the relative position of the mitotic apparatus shifts to the gc-side.

In the neuroblasts from interphase till middle prophase, a small number of microtubule arrays are observed in the nuclear core. They do not originate from the centrosome which is considered to act as an organizing center of microtubules. By electron microscopy, no microtubules associated with the centrosome are demonstrated during interphase, in early and middle prophase. The distribution pattern of microtubules indicates that the activity of the centrosome as an organizing center of microtubules is suppressed during these stages. The microtubules in the nuclear core during interphase, in early and middle prophase did not disappear after colcemid treatment which can completely depolymerize mitotic microtubules. The microtubules during these stages may be quite stable and different from the mitotic microtubules.

Summary

Microtubule distribution of grasshopper neuroblasts, which repeat unequal cell division, were demonstrated by indirect immunofluorescence microscopy. Immunoblot staining showed that both polyclonal antibody against bovine brain tubulin and monoclonal antibody against yeast tubulin used in this study cross-reacted with the grasshopper tubulin. The microtubules were organized to form the fibrous structure of the mitotic apparatus from late prophase tiil late telophase. From interphase till middle prophase, a small number of microtubules were seen distributed within the nuclear core, and were resistant to colcemid treatment which depolymerized mitotic microtubules. The size of the mitotic asters at the two spindle poles were equal from late prophase till middle anaphase, and the configuration became different at late anaphase when the spindle shifting to eccentric position was completed. The results suggest that the spindle shifting is not brought about by the differential growth of the asters between the two poles.

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要　　約

不等細胞質分裂をおこなうバッタ胚の神経原細胞における微小管の分布の変化を，微小管構成タンパク質チューブリンに対する抗体を用いた間接蛍光抗体法によって追跡した。本実験で使用したウシの脳チューブリンに対するポリクロナール抗体及び酵母菌のチューブリンに対するモノクロナール抗体ともにバッタのチューブリンと結合することがイムノブロット法によって確かめられた。微小管は late prophase から分裂装置を構成し始め，late telophase には分裂装置は完全に消失した。Interphase から middle prophase までの間は，少数の微小管が核の中央を貫く細胞質に存在しており，これらの微小管は，分裂装置微小管を完全に脱重合させる濃度のコルセミド処理に対して抵抗性を有していた。紡錘体極にある星状体の形態は，late prophase から middle anaphase までの期間は両極において差は見られなかったが，紡錘体が細胞内で偏心した位置を占めるようになった late anaphase になってはじめて両極間における相違が現れた。これらの結果は，紡錘体の偏心した位置への移動が，紡錘体両極における星状体の発達の程度の違いによって引きおこされるものではないことを示唆している。