

Development of a Differentiated Microtubule Structure: Formation of the Chicken Erythrocyte Marginal Band In Vivo

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Abstract. The microtubules of mature nucleated erythrocytes are organized into a marginal band that is confined to a single plane at the periphery and that contains essentially the same number of microtubule profiles in each individual cell. Developing erythrocytes can be isolated in homogeneous and synchronously developing populations from chicken embryos. For these reasons, these cells offer a particularly accessible system for study of the pathway leading to a specific microtubule structure in a normal, terminally differentiated animal cell. Along this developmental course, striking changes occur in the properties of the microtubules. Between the postmitotic cell and the formation of the band, a novel arrangement is found: bundles of laterally associated microtubules in each cell, coursing through the cytoplasm but not confined to the periphery. The microtubule organizing centers evident at early stages disappear by the time the band

forms. The microtubules in early cells are readily depolymerized by drugs, but that drug sensitivity is lost in the mature cells. The microtubule arrangement of mature cells is faithfully recapitulated after reversible depolymerization, while that of the immature cells is not. Finally, as the band forms, the microtubules and microfilaments increasingly become co-aligned. In sum, the microtubules of immature cells have many properties in common with those of cultured cells, but during maturation those properties change. The results suggest that lateral interactions become increasingly important in stabilizing and organizing the microtubules. The properties of marginal band microtubules, and comparable properties of axonal microtubules, may reflect differences between the requirements for cytoskeletal structures of cycling cells and terminally differentiated cells.

ONE of the central questions about cell motility arises from consideration of the different forms of the cytoskeletal elements, and of the various functions imputed to them. For example, the ultrastructural features of most microtubules are identical (Dustin, 1984), and the primary sequences of the genes that code for the major microtubule components, the alpha and beta tubulins, are highly conserved (Raff, 1984; Cleveland and Sullivan, 1985). Yet microtubules occur in a number of geometric arrangements: in mitosis, in interphase cytoplasm, and in specialized structures. They are also involved in several aspects of cell motility and maintenance of asymmetry. The origins of this diversity could lie in those covalent variations that do occur in the tubulins, or in the repertoire of minor microtubule components, or in interactions with other structural elements in the cell. Data exist supporting each of these hypotheses, although none has been demonstrated as an operative mechanism in vivo. One approach to this question is to analyze diverse microtubule organelles biochemically, functionally, and structurally, and so to identify properties they have in common and properties unique to each of them.

The mature nucleated erythrocyte contains a conspicuous example of a highly specialized microtubule structure that differs greatly from the familiar interphase array of cultured

fibroblasts. All of the microtubules in these erythrocytes are confined to a marginal band: a tight ribbon, with a fixed number of laterally aligned microtubule profiles in the equatorial plane. By both structural and kinetic evidence, the mature chicken erythrocyte lacks a microtubule organizing center (MTOC)¹ or its functional equivalent (Miller and Solomon, 1984). By contrast, all of the microtubules in cultured fibroblasts radiate individually from an MTOC, and course through all domains of the cytoplasm (Osborn and Weber, 1976; Osborn and Weber, 1977; Weber et al., 1978).

The differences between the fibroblast and erythrocyte microtubule organelles may have mechanistic implications. Based on evidence for kinetic and structural polarity of microtubules, Kirschner (1980) has argued that the presence of a common organizing center might sequester the minus ends of the microtubule from exchange with unassembled subunits, and in turn destabilize microtubules not sequestered. In that way, microtubule organization could be explained, at least in part, on thermodynamic grounds. Recent experiments on the dynamics of microtubules in vivo and in vitro support this notion (Mitchison and Kirschner, 1984; Schulze and Kirschner, 1986). However, the nature of the nucleation

1. *Abbreviation used in this paper:* MTOC, microtubule organizing center.

site is not sufficient to specify all aspects of microtubule form in fibroblasts, as shown by the *in vitro* experiments of Brinkley and co-workers (Brinkley et al., 1981). It also does not explain the properties of microtubule organelles in some terminally differentiated cells where there is no common organizing center (Chalfie and Thompson, 1979; Bray and Bunge, 1981; Sasaki et al., 1983).

In the experiments described here, we have followed the development of the marginal band. An early precursor of the mature erythrocyte must be a postmitotic cell, with an MTOC and radially oriented microtubules like those of the cultured fibroblast. We have taken advantage of the synchronous embryonic maturation of the erythrocytes to follow the development of the marginal band, and the properties that distinguish it. The results show that the microtubules in immature cells have properties similar to those of cultured cells. But, as the mature marginal band forms, those properties change. For these microtubules, and arguably for those of other differentiated cells such as neurons, different mechanisms need to be involved to explain stability and organization. An informative model is the "structured cytoplasm" described by Porter (1984); that model emphasizes the rigid organization of cytoplasmic elements that the mature erythrocyte displays.

Materials and Methods

Preparation of Erythroid Cells

To obtain immature cells, we used fertilized eggs (Spafas, Inc., Norwich, CT) held for 2 to 8 d in a Humidaire incubator (model 80; The Humidaire Incubator Co., New Madison, OH). We dissected the blastodiscs, cut the major vessels and teased the blood islands with forceps, then collected the red blood cells in PBS. After filtration through two layers of gauze, the cells were collected by centrifugation at 1,000 rpm for 5 min in a centrifuge (International Equipment Co., Needham Heights, MA) at 37°C. We removed the buffy coat along with the supernatant by aspiration, and resuspended the cell pellet in PBS plus 0.025 M glucose. This buffer supports cell viability and normal morphology for several hours at 37°–39°C. We repeated the washing two more times. Mature cells were obtained as previously described from the whole blood of adult chickens (Miller and Solomon, 1984). Incubations in drug and at low temperatures, and regrowth experiments, were all performed as for adult cells (Miller and Solomon, 1984).

Immunofluorescence and Electron Microscopy

All operations on cells were carried out at 37°–39°C, maintained in a water-bath, incubator, or constant temperature room. For immunofluorescence, cells were allowed to attach to coverslips for 10 min, then quickly washed with PM2G, a microtubule stabilization buffer (Solomon et al., 1979), and then extracted in that buffer containing 0.5% NP-40 for 5 s. They were fixed with 3.7% formaldehyde in PM2G. Attachment to glass has no apparent effect on the morphologies of the developing or mature cells, as assayed by phase and electron microscopy. Tubulin was visualized by staining with either of two antibodies, followed by appropriate second antibodies conjugated with fluorescein. One was a mouse monoclonal against tubulin, 61D, which we have described previously (Magendantz and Solomon, 1985). The second was a rabbit antibody made against the carboxyl-terminal 12 amino acids of the chicken beta-2 tubulin sequence, a gene expressed in chicken brain (Cleveland and Sullivan, 1985). The results obtained with both reagents were indistinguishable. Not shown are controls for specificity of the antibody staining. In particular, as in previous work (Solomon et al., 1979), there is no staining of cells devoid of microtubules either because they were extracted after preincubation under microtubule depolymerizing conditions or because they were extracted with buffers that do not preserve microtubules. Polymerized actin was visualized with rhodamine-labeled phalloidin. The cells were photographed on a Zeiss Photo III microscope through a 63× Planapo objective (NA 1.4), using Kodak Plus-X film, and developed in Diafine.

For electron microscopy, cells were allowed to attach to tissue culture dishes for 10 min, then fixed in 2% glutaraldehyde and 0.20% tannic acid for 45 min, and postfixed in 1% OsO₄. After washing and dehydration, the cells were embedded in Epon. Thin sections (700 Å) were viewed on a Philips 201 electron microscope.

Results

To follow the development of the marginal band *in vivo*, we have made use of the synchronous and sequestered erythropoiesis in the chicken embryo. There are several waves of development of erythrocytes in the chick embryo (Bruns and Ingram, 1973). We have studied the first wave, the primitive series, which begins at 1.5–2 d after fertilization. The cells in this lineage are the only ones in circulation until day 5. Mature cells of the primitive lineage are in circulation at day 8. The cells develop with considerable synchrony, and can be harvested either from circulation or from the erythropoietic organelles of the chick embryo, the blood islands. The first cells of the definitive series begin to appear in the circulation at day 5; these cells do not appear with such tight synchrony. The cells we isolate from the embryos resemble in detail the morphologies of the developmental stages in chicken erythropoiesis reported previously (Bruns and Ingram, 1973). Hence, the primitive series presents an excellent opportunity to study marginal band formation in a large population of identical cells.

Elaboration of the Marginal Band

The earliest time at which we are able to obtain identifiable cells from blood islands and from circulation is day 2 after fertilization. At that stage, the cells are quite spherical, and the microtubules are difficult to visualize in photomicrographs because they are in every focal plane. They are distinguishable only by focusing through the cell (Fig. 1 *a*). In some cells, the microtubules apparently are organized into spindles (Fig. 1 *a*, lower cell).

At day 3 after fertilization, we detect structures that we interpret as intermediates in the formation of the marginal band. Many of the microtubules appear to be in bundles (Fig. 1, *b* and *c*; see also Fig. 7, *a*, *c*, and *e*), so that there are fewer stained elements in these cells than in those from day 2, and they appear thicker. These bundles are not confined to the periphery or to one plane, as is the mature marginal band, but rather course throughout the cytoplasm. Focusing through the cell reveals that all the tubulin staining is organized into contiguous elements. That fact is demonstrated more clearly by transmission electron microscopy. Day 3 cells contain groups of microtubules in both longitudinal and transverse profiles within the same section (Fig. 2). We interpret these groups, which contain between 2 and 15 microtubules, as representing the bundles seen by immunofluorescence. By contrast, as shown originally by Fawcett (1959), all microtubules in sections of mature nucleated erythrocytes occur at the periphery, and show the same profile in any one section.

By day 4 after fertilization, most or all of the microtubules are in a peripheral band that is thicker and wavier than that of the adult erythrocyte (Fig. 1 *d*). By immunofluorescence it appears more intensely stained than the mature organelle, and by electron microscopy contains more microtubules (Small, 1969; Bruns and Ingram, 1973). We have seen images

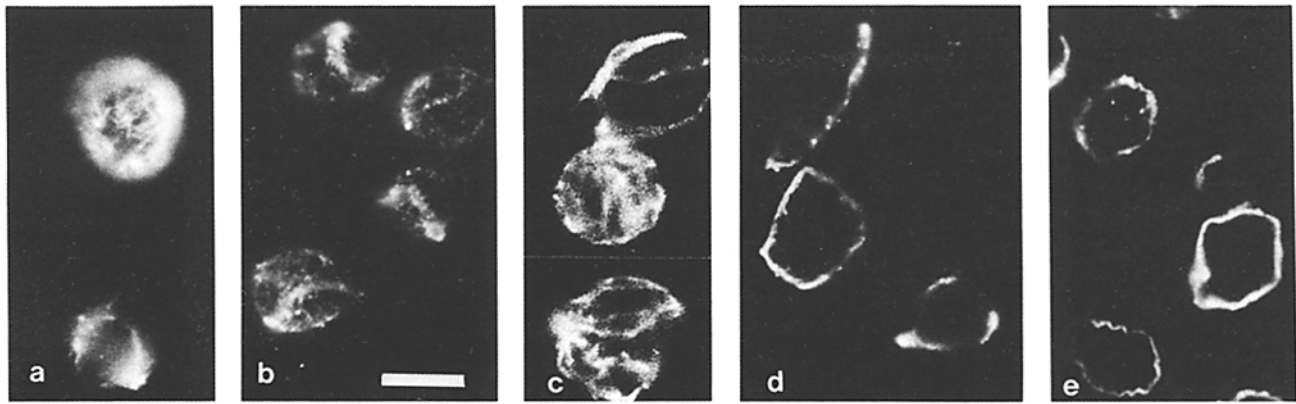


Figure 1. Microtubules in cells of the primitive series. The micrographs show cells dissected from the blood islands or the circulation of chick embryos from different stages, extracted and fixed in microtubule stabilization medium, then stained with antibodies against tubulin. (a) Day 2 cells, showing diffuse array in spherical cells (*top*) and an array suggesting a mitotic spindle (*bottom*). (b and c) Day 3 cells, showing microtubules condensed into several bundles. (d) Day 4 cells, showing most microtubules in a single peripheral band. (e) Day 5 cells, again showing marginal band. The differences in staining intensity along the bands in *d* and *e* are due to the band passing in and out of the plane of focus, not to a change in the tubulin density at different domains of the band. In *c*, all the cells were in one microscope field, but the space between them has been cropped so they would all fit in the format. Bar, 7 μm .

of loosely packed marginal bands less condensed than these, images also detected by others (Murphy et al., 1986) and compared to a wreath, but they are extremely rare among these embryonic populations. At day 5, all of the cells in circulation contain fully formed marginal bands (Fig. 1 *e*). After day 5, the circulation includes cells of both the primitive and definitive series of development, so that the populations are no longer uniform.

The Participation of MTOCs

Previously, we described a set of results suggesting that at least a typical MTOC is not present in adult chicken erythrocytes (Miller and Solomon, 1984). We were unable to find such a center by ultrastructural analysis, by immunological probes, and by observing the kinetics and intermediates of marginal band reformation in vivo after depolymerization. Other cells with marginal bands from invertebrates do have apparent MTOCs (Nemhauser et al., 1983). Developing erythrocytes in the marrow of anemic chickens display a microtubule pattern consistent with the presence of MTOCs, although they are not demonstrated directly (Murphy et al., 1986). However, in maturing embryonic erythrocytes, MTOCs can be clearly visualized. The cell shown in Fig. 3 *a* is from a day 4 embryo. Its microtubules are mostly, but not exclusively, in a peripheral band. Those microtubules that are not in the band appear to originate at a single site at the bottom of the micrograph, and to show a radial orientation similar to that of interphase microtubules frequently portrayed in tissue culture cells (Osborn and Weber, 1976). A phase image of the same cell (Fig. 3 *b*) shows a dark structure at precisely the position of the immunofluorescent focal point. A correspondence between fluorescence pattern and pairs of phase-dense structures has been used as evidence for MTOCs in studies by others (Nemhauser et al., 1983). (Similar dark bodies can also be seen in mature chicken erythrocytes, although their identification as MTOCs is not supported by staining with anti-tubulin [Pillus, L., unpublished observations]). The MTOCs in developing cells are likely associated with centrioles. The micrograph in Fig. 4

shows a thin section through an interphase cell from day 2, and includes a cross-section of a centriole, and several microtubule profiles close to it. Therefore, it seems likely that at least some of the microtubules of early erythrocytes are associated with an MTOC. However, in these developing chicken erythrocytes, the MTOCs are close to the periphery as are the majority of the microtubules. By contrast, in cultured cells, the MTOCs are located close to the cell center. The images shown in Figs. 3 and 4 occur frequently through day 4 of development, but we have not seen evidence for MTOCs at day 5 and beyond. We do not know the precise time at which the centriole structures disappear, nor have we detected any intermediates in their disappearance.

Loss of Sensitivity to Microtubule Depolymerizing Drugs

Microtubules of immature erythrocytes are completely depolymerized by standard concentrations of microtubule depolymerizing drugs. In 0.1 $\mu\text{g}/\text{ml}$ nocodazole, all the microtubules in cells from day 3 embryos are disrupted within 90 min, as assayed by immunofluorescence (Fig. 5, *a* and *b*). That result is confirmed by biochemical techniques (Solomon et al., 1979); there is essentially no assembled tubulin in a population of cells treated in this fashion (data not shown). With increasing age, the microtubules become resistant to drug-induced depolymerization. In populations of circulating cells from day 8 embryos, different stages of development are represented because of the contribution of both the primitive and definitive series. The cells that represent late stages of the primitive series, marked by characteristically large and less condensed nuclei, are essentially stable to nocodazole. However, microtubules in the cells of the definitive series, which have smaller and darker nuclei, are still disrupted by the drug (Fig. 5, *c* and *d*). Adult cells are completely refractile to long incubations at high concentrations of nocodazole. At all stages of development, the microtubules depolymerize in cells incubated at 4°C for 1 h (Miller and Solomon, 1984; and data not shown).

The resistance to microtubule depolymerizing drugs in

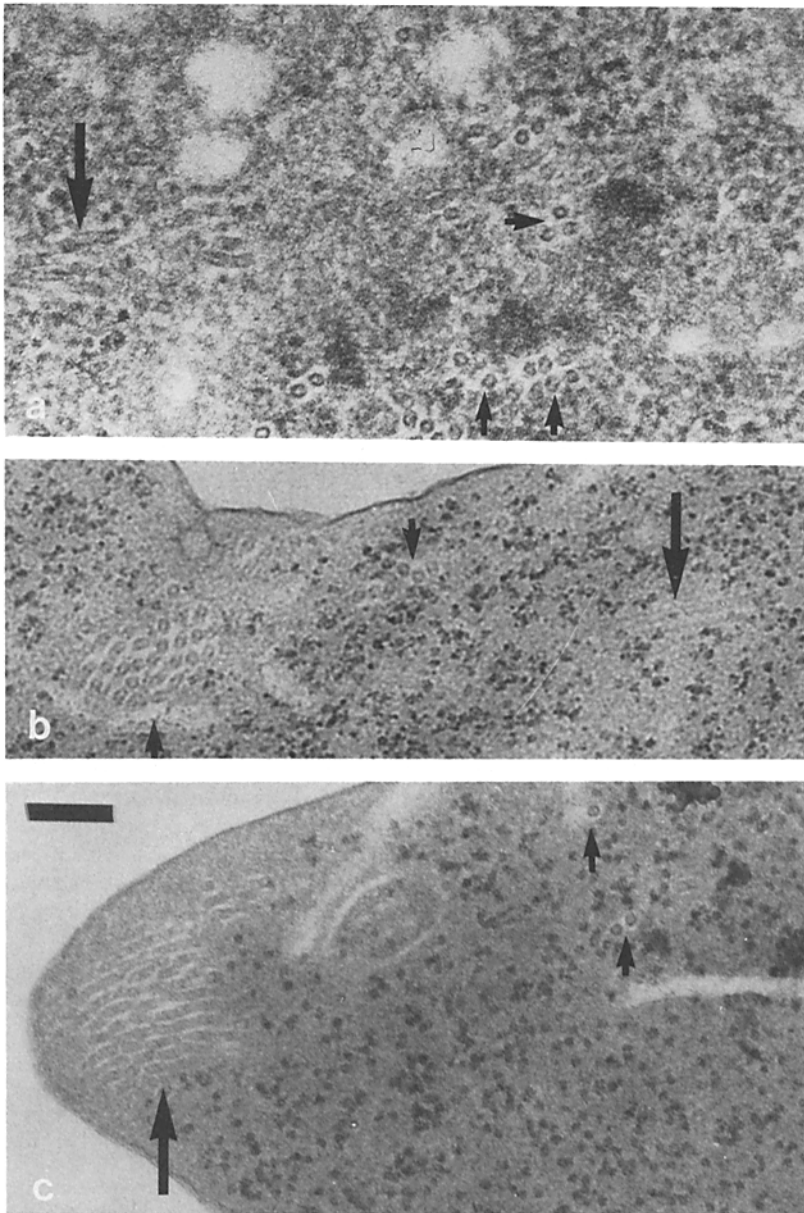


Figure 2. Microtubular bundles in day 3 cells. The electron micrographs show bundles of microtubule profiles in sections through day 3 cells. Both longitudinal profiles (*large arrows*) and cross-sectional profiles (*small arrows*) are visible. In mature cells, only one sort of profile is seen in any one section. Bar: (a and b) 0.10 μm ; (c) 0.12 μm .

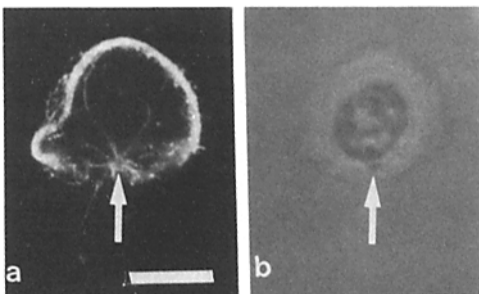


Figure 3. MTOCs in immature erythrocytes. The micrographs show a day 4 cell, extracted and fixed. The anti-tubulin staining (a) shows most of the microtubules in a band structure, but some off the band and radiating from a single center (*arrow*). A phase micrograph of the same cell (b) shows a dark object (*arrow*) coincidental with the apparent focal point of those microtubules not in the band. Bar, 6 μm .

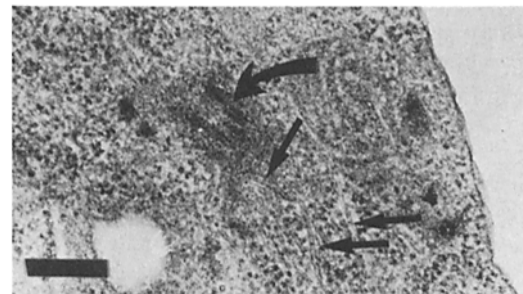


Figure 4. Centrioles in immature erythrocytes. The electron micrograph shows a cross-section through a centriole (*large arrow*) and numerous microtubule profiles in the same region (*small arrows*) of a cell isolated from a day 2 embryo. The centriole here, like the putative MTOC in Fig. 3, is closer to the periphery than MTOCs in most cultured cells. We find no profiles of centrioles in mature cells of the chick or of the adult chicken. Bar, 0.2 μm .

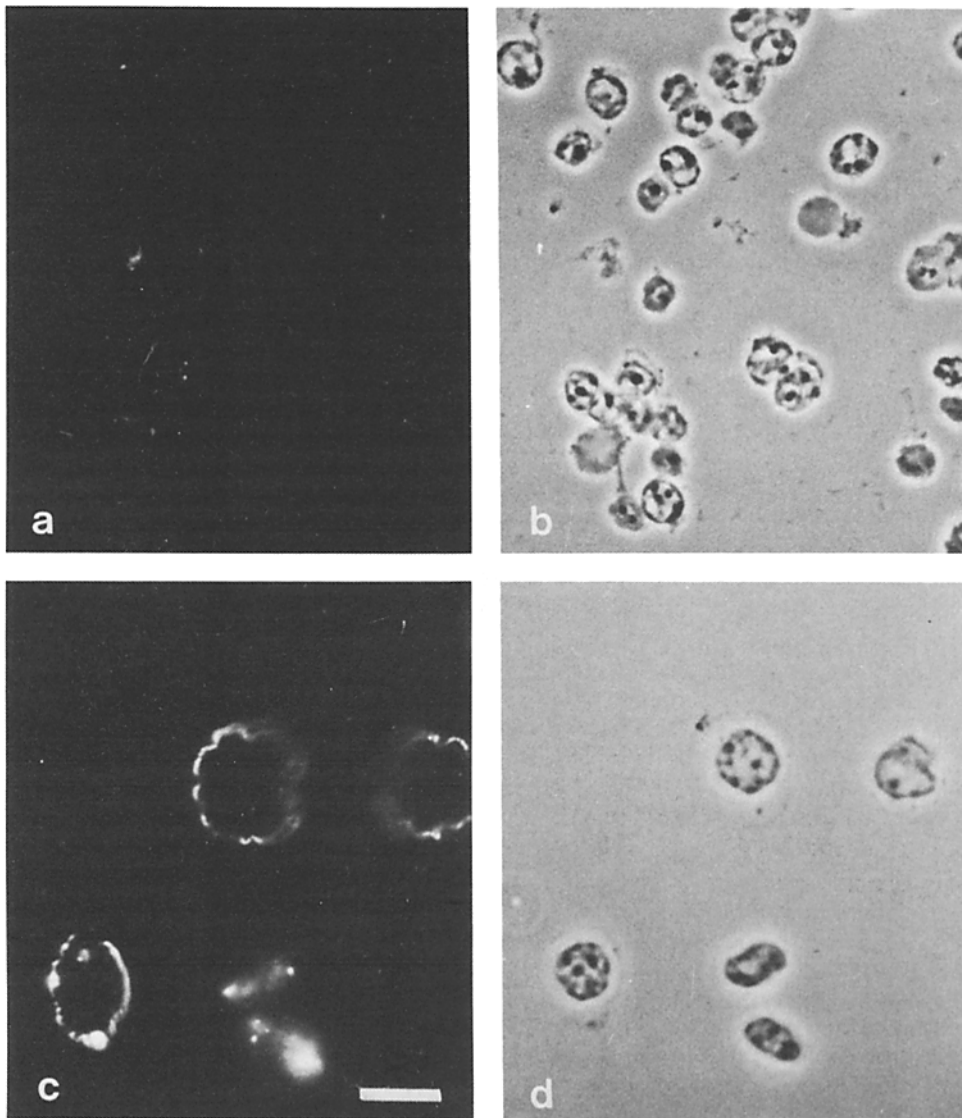


Figure 5. Sensitivity to microtubule depolymerizing drugs. Cells from day 3 (*a* and *b*) and day 8 (*c* and *d*) were incubated for 60 min with 0.1 $\mu\text{g/ml}$ nocodazole, then extracted and fixed for immunofluorescence (*a* and *c*) and phase microscopy (*b* and *d*). Virtually all of the microtubules are depolymerized in the day 3 cells. In the population from day 8, the cells with large nuclei, and which are mature cells of the primitive series, still retain all of their tubulin staining. The two cells with smaller, denser nuclei in this field probably represent less mature cells of the definitive series. Their marginal bands are disrupted, although there remain beads of stainable material. These beads also stain with other antibodies, and so the staining is likely to be nonspecific. Bar: (*a* and *b*) 10 μm ; (*c* and *d*) 6 μm .

mature cells could be due to a permeability barrier to the drug itself, arising from changes in the property of the plasma membrane; this possibility is testable. We incubated mature cells in the cold in the presence of 0.1 $\mu\text{g/ml}$ nocodazole, then shifted the cells to 39°C for 1 h. As previously reported (Miller and Solomon, 1984), the marginal band is completely reformed upon rewarming in the absence of drug. However, in the presence of nocodazole, the marginal band does not reform, even after prolonged incubation. This experiment demonstrates that the drug resistance is most likely a characteristic of the microtubule organelle itself, rather than other properties of the cell. Similar results have been reported for the microtubules in the mature erythrocytes of the mature dogfish, using colchicine as the microtubule depolymerizing drug (Joseph-Silverstein and Cohen, 1984).

Reformation of the Marginal Band after Depolymerization

The marginal band of the mature erythrocyte will reform precisely after cold depolymerization and rewarming. Both quantitatively (the number of microtubule profiles in the

marginal band) and qualitatively (the position, shape, and apparent length of the microtubules), the marginal band regrown in such a fashion is indistinguishable from that of untreated cells (Miller and Solomon, 1984). However, when cells at earlier stages of development are carried through the same experiment, the marginal band is not recapitulated with such precision. For example, day 5 cells have all their microtubules organized into a marginal band structure, at least at the resolution of the light microscope (see Fig. 1 *e* above). Upon chilling and rewarming, most of the tubulin staining is again in a peripheral bundle. However, in every cell there are microtubules distinct from that structure (Fig. 6 *b*). In untreated day 5 cells, stained and photographed under the same conditions, no microtubules off the band are visible (Fig. 6 *a*). The differences in intensity at different positions along the band are the result of the band moving in and out of the focal plane, and are not due to variations in the actual composition of the band.

Relationship between Microtubules and Microfilaments

The polymerized actin in immature cells can be visualized

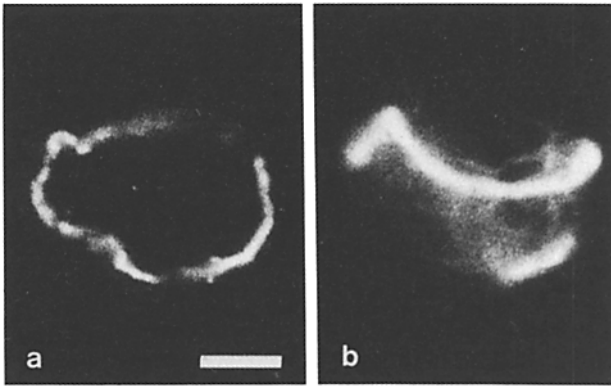


Figure 6. Regrowth of microtubules after cold depolymerization in day 5 cells. Cells from day 5 embryos are incubated at 0°C for 90 min, then incubated at 39°C for 60 min. Some of the microtubules in the cells treated this way are visualized as individual fibers independent of the marginal band (*b*), while those of control 5-d cells, which have not been depolymerized, are all in the marginal band (*a*). Bar, 3 μ m.

by staining with rhodamine-labeled phalloidin. In the youngest cells, from day 2, the actin and tubulin patterns are diffuse. However, discrete patterns are apparent in day 3 cells, when the microtubule bundles become well defined. Fig. 7 shows the microtubule (panels *a*, *c*, and *e*) and actin (panels *b*, *d*, and *f*) patterns from three day 3 cells. The actin staining and tubulin staining are concentrated in the same domains of the cells. In some regions, the two staining patterns are very similar. But in every cell there are areas stained by anti-tubulin antibodies that are not stained with phalloidin, and the converse is also true. However, in mature erythrocytes the microtubules and microfilaments coalign to a considerable extent (Fig. 8, *a* and *b*). These mature cells are stained under the same conditions as the day 3 cells in Fig. 7, and control experiments (not shown) demonstrate that the coincidental signals are not due to crossover between the fluorescein and rhodamine detection pathways. This correlation is best revealed by focusing through individual cells. It can be represented in micrographs by including several cells in a field. The marginal band goes in and out of the plane of focus, because the cells stick to the coverslip with slightly different pitch, and because there is some twist in the marginal bands themselves. When the rhodamine-phalloidin labeling of the same cells is visualized, the domains of the actin staining that are in and out of the focal plane are clearly seen to coincide with those of the marginal band. At least at the level of resolution of the light microscope, the two structures colocalize. We have been unable to identify a stage of development in which only the actin or only the microtubules are at the periphery.

Discussion

The experiments described above document the changes that occur in the cytoskeleton of chicken red blood cells during embryonic development. At the beginning of this lineage are the postmitotic cells, with individual microtubules radiating from microtubule organizing centers. At the end is the mature erythrocyte, in which all microtubules are confined to a single structure, an equatorial ribbon or band with a pre-

cise number of laterally arrayed components. Over this same period of ~ 3 d, the characteristics of the microtubules, and their relationship to other cell components, are changed as well.

Intermediates in Marginal Band Formation Suggest the Influence of Lateral Interactions

The microtubules begin as apparently individual elements, coursing through all domains of the cytoplasm of the spherical immature cells. At later stages, by day 4, they are primarily confined to the marginal band. Others have noted that the final, compact band is preceded by a looser array of microtubules in a band-like pattern in the bone marrow of anemic chickens (Murphy et al., 1986) and we have seen similar structures in embryonic cells, although they are very rare. At day 3 of development, however, another and presumably earlier intermediate in marginal band formation is evident. In cells at this stage, the microtubules are gathered into several bundles which run through several different domains of the cytoplasm (Figs. 1, *b* and *c*, and 2). The simplest interpretation of the existence of these bundles is that the developing erythrocyte goes through a stage where lateral microtubule-microtubule interactions become important, and that those interactions lead to the eventual coalescence of all the microtubules in the cell. An alternative pathway might involve complete depolymerization of the microtubules, and their reformation at the position of the marginal band. We have seen none of the intermediates that would be predicted in such a pathway were this rearrangement to pro-

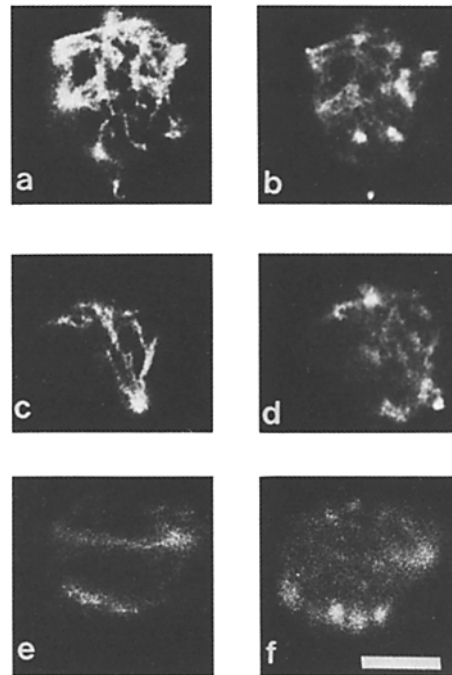


Figure 7. Double staining of day 3 cells for tubulin and polymerized actin. The micrographs show double staining of three cells from day 3 embryos with antibodies against tubulin (*a*, *c*, and *e*) and rhodamine-labeled phalloidin (*b*, *d*, and *f*). The tubulin staining, like that in Fig. 1 *b*, shows that the microtubules are largely collected into bundles. The actin staining is quite patchy, and few, if any, filamentous structures are seen. The two patterns are clearly similar in places but not coincidental. Bar, 5 μ m.

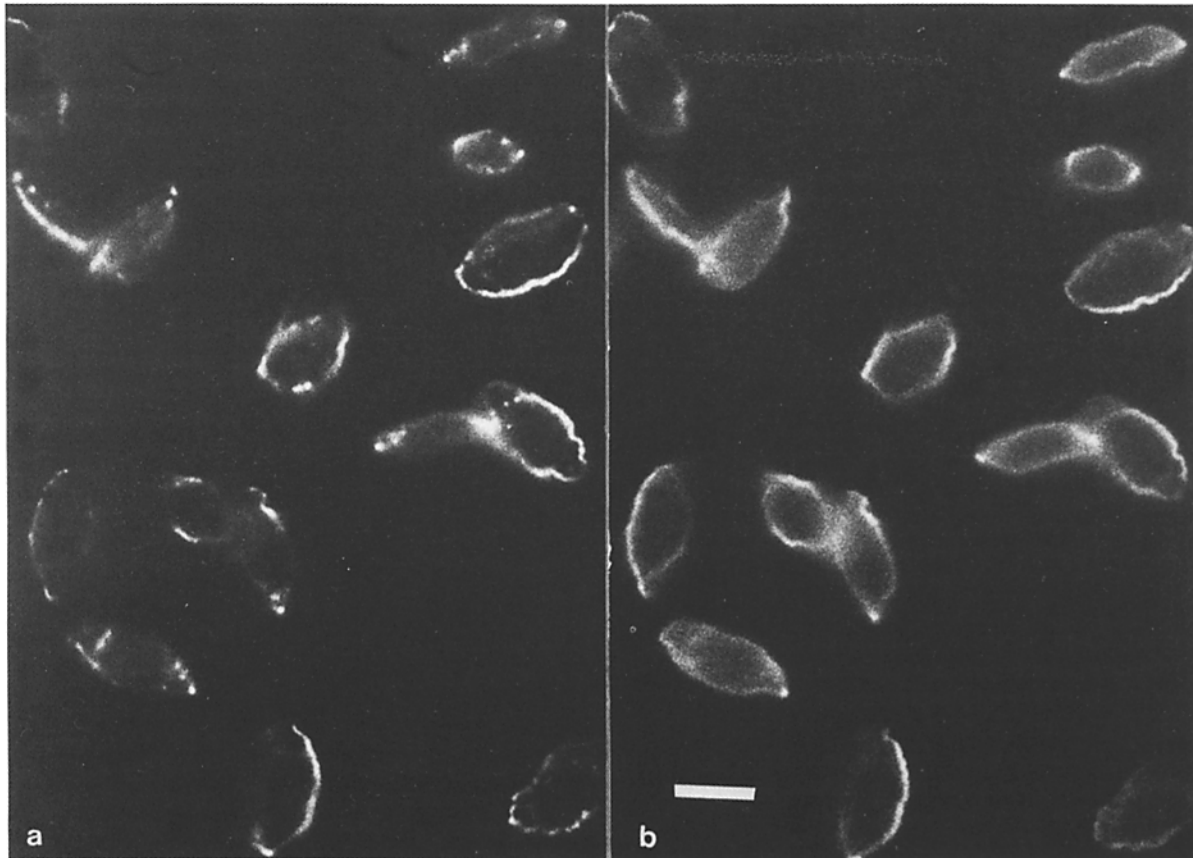


Figure 8. Double staining of adult erythrocytes for tubulin and polymerized actin. The micrographs show a field of cells stained as in Fig. 7 with antibodies against tubulin (a) and rhodamine-labeled phalloidin (b). Comparison of the patterns in several cells shows that the actin and tubulin staining patterns are confined to the periphery, and both go in and out of the plane of focus in the same places along the cell as does the tubulin staining. Control experiments, and results of other experiments like the one shown in Fig. 7, demonstrate that the coincidence of staining is not due to crossover between the channels, or nonspecific sticking of the antibodies. Bar, 5 μ m.

ceed in concerted fashion; if it were to occur in gradual fashion, the intermediates might be difficult to detect.

A pathway involving bundling of microtubules is different from the one inferred by regrowth experiments performed in this laboratory on mature cells. In intact cells, microtubules repolymerizing after cold-mediated depolymerization grow as if from just one or two ends rather than in bundles, and stop growing when the original number is reached (Miller and Solomon, 1984). It is also possible to recreate this organelle *in vitro*. We added purified calf brain tubulin to detergent extracted cells that contain no microtubules and are devoid of endogenous tubulin (Swan and Solomon, 1984). The polymerization is in the appropriate place in the cell, and the microtubules have the appropriate shapes; and, as in the *in vivo* regrowth, the regrowth stops when the original number of microtubules is reached. This *in vitro* reformation of the marginal band requires neither endogenous tubulin nor the plasma membrane of the cell. Essentially pure calf brain tubulin is a sufficient substrate for marginal band formation *in vitro*. In both of these regrowth experiments, there is no evidence for interaction between microtubule elements; rather, the crucial interactions are between each independent microtubule and the rest of the cytoskeleton. These results suggest that the interactions that are sufficient to specify a marginal band in the mature erythrocyte may not reflect ac-

curately the interactions responsible for forming the mature organelle *in vivo*. Studies of marginal bands from other organisms suggest that the microtubules are cross-linked to one another. For example, marginal bands can be isolated intact and apparently free of other cytoskeletal material from dogfish erythrocytes (Cohen, 1978) and from newts (Bertolini and Monaco, 1976). But using the approaches described in those experiments, and several modifications of them, we have been unable to isolate chicken erythrocyte marginal bands.

It is also useful to compare the results of regrowth experiments with the mature erythrocyte to the outcomes of very similar experiments done earlier with fibroblasts (Brinkley et al., 1981). When detergent extracted fibroblasts devoid of microtubules are used as a substratum for microtubule assembly the regrown microtubules are quite straight, not wavy as they are in the interphase cytoplasm of these cells, and they extend past the cell perimeter originally occupied by the plasma membrane. In addition, the assembly on the fibroblast cytoskeleton preparation initiates from the MTOCs, while the assembly on the erythrocyte preparations shows no such defined initiation sites. Therefore, the fibroblast preparations have the capacity to nucleate microtubule assembly but not to specify their position and form.

MTOCs May Be Involved in the Establishment but Not the Maintenance of the Mature Marginal Band

Immature erythrocytes clearly contain a microtubule organizing center, as demonstrated here by both light and electron microscopy (Figs. 3 and 4). Such a structure is present in other cells with a marginal band (Nemhauser et al., 1983), and evidence for one in developing chicken red blood cells has been inferred from immunofluorescence patterns (Murphy et al., 1986). However, this structure is absent from mature cells of the primitive series, as well as from mature circulating cells of the adult chicken (Miller and Solomon, 1984). Whether some unidentified structural element takes its place is unknown, but at the least a common origin for microtubules is not required to maintain the marginal band. This situation is reminiscent of another terminally differentiated cell, the mature neuron. In the axons of those cells, microtubules appear to start and stop along the length of the process, and also do not insert into a common organizing center (Chalfie and Thompson, 1979; Bray and Bunge, 1981; Stevens, 1983). One difference between these two situations is that in the axons, the pool of unassembled tubulin is apparently quite small, while in the erythrocyte it accounts for ~80% of the total cell tubulin (data not shown). But in both cases, some interaction other than sequestration of minus ends with an MTOC as usually defined must be responsible for stabilization of the assembled structures. It is possible that the lateral interactions between microtubules postulated above, or interactions between microtubules and other cytoskeletal elements, could supplant the MTOC.

Marginal Band Microtubules Lose Sensitivity to Depolymerizing Drugs during Development

The microtubules of the maturing erythrocytes acquire resistance to microtubule depolymerizing drugs. This resistance may reflect a lack of dynamic interchange between soluble and assembled pools of tubulin, a possibility which is difficult to document by an independent technique. At any rate, resistance is detected after the MTOC has disappeared and as the microtubules are condensing into a band. A similar resistance to drug depolymerization occurs among axonal microtubules (Black and Greene, 1982). One interpretation of this result is that the microtubules become increasingly stabilized with time; that stabilization, however it is imposed, might also explain why an MTOC is no longer required to preserve the integrity of this organelle. That lateral stabilization can supplant the role of MTOCs is supported by the demonstration that injection of a microtubule-associated protein into fibroblasts stabilizes microtubules not connected to an organizing center (Drubin and Kirschner, 1986).

Microtubule Patterns Become Less Plastic during Development

Day 5 cells have their microtubules in a band, and no microtubules elsewhere. However, after depolymerization by either cold or drug, followed by repolymerization, some of the microtubules reform away from the band. This is not the result we obtained observing microtubule regrowth in mature cells after cold-mediated depolymerization (Miller and Solomon, 1984). In those experiments, no microtubules could be found that were not in the marginal band domain, even at

early times of regrowth. These results suggest that, along with the increased stability of the mature band as discussed above, there is a loss of plasticity in these cytoskeletons with maturation.

Microtubules and Microfilaments Coalign at the Marginal Band during Development

The pattern of actin staining in developing erythrocytes also changes with development. In very early stages, the pattern is diffuse. As the bundles of microtubules form, the actin staining also coalesces into discrete areas. That staining frequently is similar to that obtained with anti-tubulin antibodies in the same cell; there are clear domains of overlap. There are also elements of the staining pattern for each that does not coincide with staining by the other. In contrast, the microtubules and polymerized actin of the mature erythrocytes are closely aligned, so much so that their immunofluorescent patterns are virtually superimposable in every cell. The functional significance of this arrangement is not known, but it may reflect special requirements for the cytoskeleton of these cells that are subjected to exceptional distortion and changes in pressure as they move through capillaries, but need no longer carry out mitosis or self-powered motility (Joseph-Silverstein and Cohen, 1984).

Models for the Cytoskeleton of the Mature Erythrocyte

The properties of the mature marginal band are difficult to explain using models that rely exclusively upon events at microtubule ends to control dynamics and morphogenesis. These microtubules are more stable, they lack MTOCs, and they apparently are involved in important lateral interactions that can control their shape and their position. As pointed out above, at least some of these properties are shared by microtubules in another terminally differentiated cell, the neuron. Axonal microtubules are also more stable than those of cultured cells as assayed by the same criterion applied here, their sensitivity to depolymerizing drugs. They also appear to lack organizing centers; axonal microtubules are discontinuous, and do not insert into a common site. They could be "capped" individually (Chalfie and Thompson, 1979), although there is no direct evidence for such a structure. It is also clear that axonal microtubules are involved in important lateral interactions; for example, there is a characteristic spacing between microtubules in axons, and they appear to be transported in concert with other structural elements of the axon.

At least for these two microtubular organelles, stability and interaction apparently have superceded the "dynamic instability" (Schulze and Kirschner, 1986) of microtubules in cultured fibroblasts. Teleologically, such a transformation can be explained by the different requirements placed on the cytoskeleton of a cell that has reached its final shape as opposed to one that must still remodel itself entirely to undergo mitosis. In the former situation, the ability to rapidly depolymerize microtubules is no longer relevant. Perhaps the mechanisms that have been invoked to explain microtubule morphogenesis in cultured cells will not explain satisfactorily the properties of these stabler structures. One alternative model is that described by Porter (1984) as a structural cytoplasm. The features of that model most relevant to the marginal band are the nonrandom localization and configu-

ration of cytoplasmic organelles and the capacity to return to a particular configuration after perturbation. Probably the most dramatic example of this property of the cytoplasm is presented by studies on the chromatophore. These cells can move pigment granules through cycles of dispersion and aggregation, movements that involve significant distortions of the cytoskeleton. Yet individual pigment granules return to the same position after each cycle with remarkable precision (Porter and McNiven, 1982). Other cells can also recapitulate their original morphologies after reversible microtubule disassembly (Solomon, 1980). These characteristics of the marginal band and perhaps of other normal, differentiated cell structures can also be thought of as the quaternary structure of a cytoskeletal organelle. In the mature nucleated erythrocyte, with the accessibility of its precursors and the relative simplicity of its protein complement, we may have the system for solving that quaternary structure in detail.

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