Reformation of the Marginal Band of Avian Erythrocytes In Vitro Using Calf-brain Tubulin: Peripheral Determinants of Microtubule Form

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ABSTRACT The microtubules of nucleated erythrocytes form an extraordinary structure: they are organized into a marginal band at the periphery of the cell. This unusual organelle, recurring in detail in each cell, provides an excellent opportunity to study the determinants of microtubule form. We have been able to reform the marginal band, using detergent-extracted erythrocytes that have been depleted of microtubules in vivo and phosphocellulose-purified tubulin from calf brain. We find that detergent-extracted cytoskeletons incubated under these conditions again have microtubules, and that the pattern of these microtubules recapitulates several features of the intact marginal band. In particular, most of the microtubules after regrowth are located in a band at the periphery of the cell, and curve to form an ellipse. These results support the hypothesis that the specification of microtubule location and shape in these cells is governed by determinants that reside at the periphery of the cell.

The pattern of microtubules in a cell is a major aspect of the assumption and maintenance of cell polarity and morphology. Although most microtubules have essentially the same ultrastructure and are composed of highly conserved alpha- and beta-tubulins, their cellular patterns are diverse. The shape, number, and location of microtubules vary dramatically between cells and even within a single cell through the course of the cell cycle. We are interested in understanding how the pattern of microtubules in a cell is specified. That pattern might arise from the properties of the nucleating sites, or microtubule-organizing centers (MTOCs)[1] acting at the ends of the microtubules to determine their characteristics, such as length or number. Alternatively, specific lateral interactions with other cell components, determined by different tubulins or different associated proteins or both, may specify microtubule configuration. Evidence consistent with each of these possibilities—special MTOCs (3), special associated proteins (6, 10, 22), and special tubulins (21)—has been presented from several laboratories.

To explore this question, we are studying the mature avian erythrocyte, a normal cell type with an extraordinary microtubule structure. All its microtubules are located at the periphery of the cell in one plane, bundled together in a structure referred to as the marginal band. The number of microtubule profiles in the band is also controlled and is characteristic of the cell type (7). In an earlier paper we followed the reformation of the marginal band in intact cells after depolymerization, in an in vivo reassembly reaction. We showed that the band was quantitatively and qualitatively reformed in a time-dependent reaction. In addition, we found no evidence for the participation of an MTOC in the initiation of microtubule assembly. Rather the data suggested that dispersed, peripheral determinants of microtubule location and shape specify the pattern of microtubules in these cells (12).

In this paper we report the successful reiteration of the microtubule pattern in erythrocytes by in vitro assembly. This in vitro procedure, developed by Brinkley and his colleagues (3, 11, 17) for mammalian cells, uses exogenous, phosphocellulose-purified tubulin from calf brain, which is added to detergent-extracted cytoskeletons of erythrocytes depleted of microtubules. We find that the purified tubulin will indeed form microtubules in the presence of the extracted cytoskeletons, but not in their absence. The form and location of these microtubules recapitulated the marginal band. These results demonstrate that the unusual conformation of the microtubules in these cells is independent of specific eryth-

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

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rocyte tubulins, but rather can be specified by cold-stable peripheral determinants.

MATERIALS AND METHODS

Preparation of Erythrocyte Cytoskeletons: Erythrocytes were prepared from whole blood using the same protocol as described previously (12). After 90-min incubation of whole cells at 0°C or 39°C, the cells were plated on grid-coverslip assemblies. These assemblies were rinsed in phosphate-buffered saline, rinsed once in a microtubule-stabilizing buffer PM2G PIPES; 10^{-3} M magnesium sulfate; 2×10^{-3} M EGTA; 2 M glycerol; phenylmethylsulfonyl fluoride; aprotinin; pH 6.9 (20), placed in a petri dish and immediately covered with PM2G plus 0.1% Nonidet P-40, and 5 μg/ml taxol (18, 19). The cells were extracted in this buffer for 5 min, extracted again for 2 min in the same buffer, then extracted again for 2 min in PM2G without taxol.

Preparation of Purified Calf Brain Tubulin: Calf brain microtubule protein was prepared by two rounds of temperature-dependent assembly in the absence of glycerol (2). To prepare tubulin incompetent to self-assemble (22), we thawed 4–8 mg of twice-cycled microtubule protein from −80°C, and put it through one more cycle of assembly. The assembled microtubules were pelleted, and resuspended with a hypodermic needle in 0.35 ml of column buffer (0.05 M PIPES; 10^{-3} M EGTA; 5×10^{-4} M magnesium chloride; 10^{-4} M GTP) and incubated on ice for 30 min. The resuspended protein was loaded onto a 1-ml phosphocellulose column, and the flow-through, containing tubulin free of associated proteins, was eluted using column buffer. Approximately 0.6-ml fractions were collected directly into tubes containing 0.2 ml of adjusting buffer (8 M glycerol, 0.25 M PIPES, 5×10^{-3} M EGTA, 0.025 M magnesium sulfate, 4×10^{-3} M GTP). The adjusting buffer served to transfer the tubulin from the column buffer into microtubule-stabilizing buffer PM2G, at 10^{-3} M GTP. Aliquots of each fraction were immediately assayed for protein content using the Bradford Protein Assay (Bio-Rad Laboratories, Richmond, CA). The peak fraction was used either neat or diluted into PM2G-GTP. The concentrations of purified tubulin used ranged from 0.75 to 4.0 mg/ml; the usual concentration employed was 1–2 mg/ml. The purified tubulin was free of most co-assembling proteins as determined by silver stained (9, 13) SDS PAGE (Fig. 1). The activity of the purified tubulin decreased rapidly with time after elution from the phosphocellulose column. It was therefore critical that the tubulin be used within 10 min of its elution.

Regrowth of Marginal Band Microtubules: Coverslips bearing the extracted cytoskeletons from cells that had been incubated in vivo at 0°C were blotted, and 50 μl of purified tubulin in PM2G-GTP was added to each coverslip. Control coverslips were incubated in 50 μl PM2G-GTP alone. The coverslips were then incubated at 35°C for 10 min; other time points between 5 and 20 min were taken, depending on the experiment. After incubation, coverslips were blotted to remove excess tubulin, then dipped vigorously five times into a beaker of PM2G, to wash away unpolymerized tubulin. In some experiments the cells were incubated for 2 min in PM2G containing taxol to help preserve microtubules (23). Coverslips were then rinsed twice more in 50 ml of PM2G each, and fixed in PM2G containing 3.7% formaldehyde and 0.5% glutaraldehyde at room temperature for 30 min. Coverslips were prepared for electron microscopy as previously described (12), except that the osmium tetroxide concentration was 0.5%.

Scoring Samples: To obtain a representative sampling of each grid, we photographed whole mounts erythrocytes as they were encountered independent of whether they contained microtubules. The extent of regrowth of the marginal band of microtubules was quite variable from one section of a grid to another, while adjacent cells generally showed similar levels of regrowth. Photographs were usually taken at 7,000 magnification, and then printed at a further 2.9-fold enlargement. Each print was inspected for microtubule profiles by each of the authors, and the number of microtubules counted at three or more sites at the periphery.

RESULTS

In Vitro Regrowth of the Marginal Band Is Efficient and Reproducible

After detergent extraction in microtubule stabilization buffer, the microtubules of the marginal band are clearly visible by whole mount electron microscopy (Fig. 2*a*). In micrographs at suitable magnifications, every microtubule in the marginal band can be counted (Fig. 2*b*). The marginal band is cold labile in vivo (12), so that after incubation at 0°C

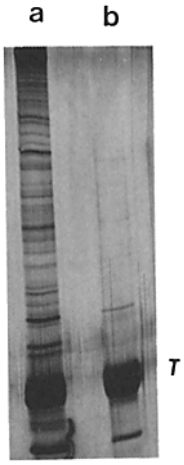

FIGURE 1 SDS PAGE analysis of (*a*) whole microtubule protein after three cycles of temperature-dependent assembly-disassembly and (*b*) the flow-through fraction of that material applied to a phosphocellulose column. The protein is visualized by silver stain. *T* indicates the mobility of the tubulin bands.

for 90 min, usually 97% of the cells have no visible microtubules (Fig. 2, *c* and *d*). However, when these same cytoskeletons are incubated with purified tubulin, microtubules again are seen in the cells. No microtubules are found on the grids when the tubulin is incubated alone under identical conditions. The reaction is efficient: >80% of the cells display one or more microtubules after incubation with tubulin (Fig. 3). The reaction appears to be dependent on the concentration of added tubulin. At lower concentrations, fewer cells have microtubules; at higher concentrations, the number of cells with microtubules, and the number of microtubules per cell, increase. Below ~1 mg/ml concentration of added tubulin, no regrowth occurs. The reaction is highly reproducible from experiment to experiment using the conditions described here. Taxol enhances the extent of the regrowth reaction when included in the first extraction, although it is not absolutely required.

Regrown Marginal Bands Recapitulate the Original Pattern of Microtubules

The regrown microtubules are located primarily at the periphery of the cells, where the marginal band was originally found. Representative images are shown in Fig. 4. Approximately 5% of the cells have no microtubules at all after incubation with tubulin, and look identical to cells incubated without tubulin. Another 10% of the cells have less than one entire microtubule in the cell: either they have a long microtubule that does not circle the entire cell or they have one to four short microtubule fragments somewhere on the periphery or both (Fig. 4*a*). In 35% of the cells the marginal band is smooth and continuous, and few microtubule ends are visible (Fig. 4, *b* and *c*). This is the usual appearance of cells whose marginal bands were regrown in vivo. Approximately 40% of the cells have a discontinuous marginal band, as shown in Fig. 4*d*, where most microtubules are at the perimeter of the cell, but the band consists of many short microtubules, often aligned imperfectly with respect to the long axis of the cell. We have never found such images among marginal bands regrown in vivo. The remainder of the cells, ~10%, are similar to the cell in Fig. 4*e*, where microtubules have formed almost everywhere. Cells such as this one showing heavy regrowth will often have microtubules in the cytoplasm between the marginal band and the nucleus, and running from cell to cell. However, even in these cells the bulk of the microtubules are located at the periphery.

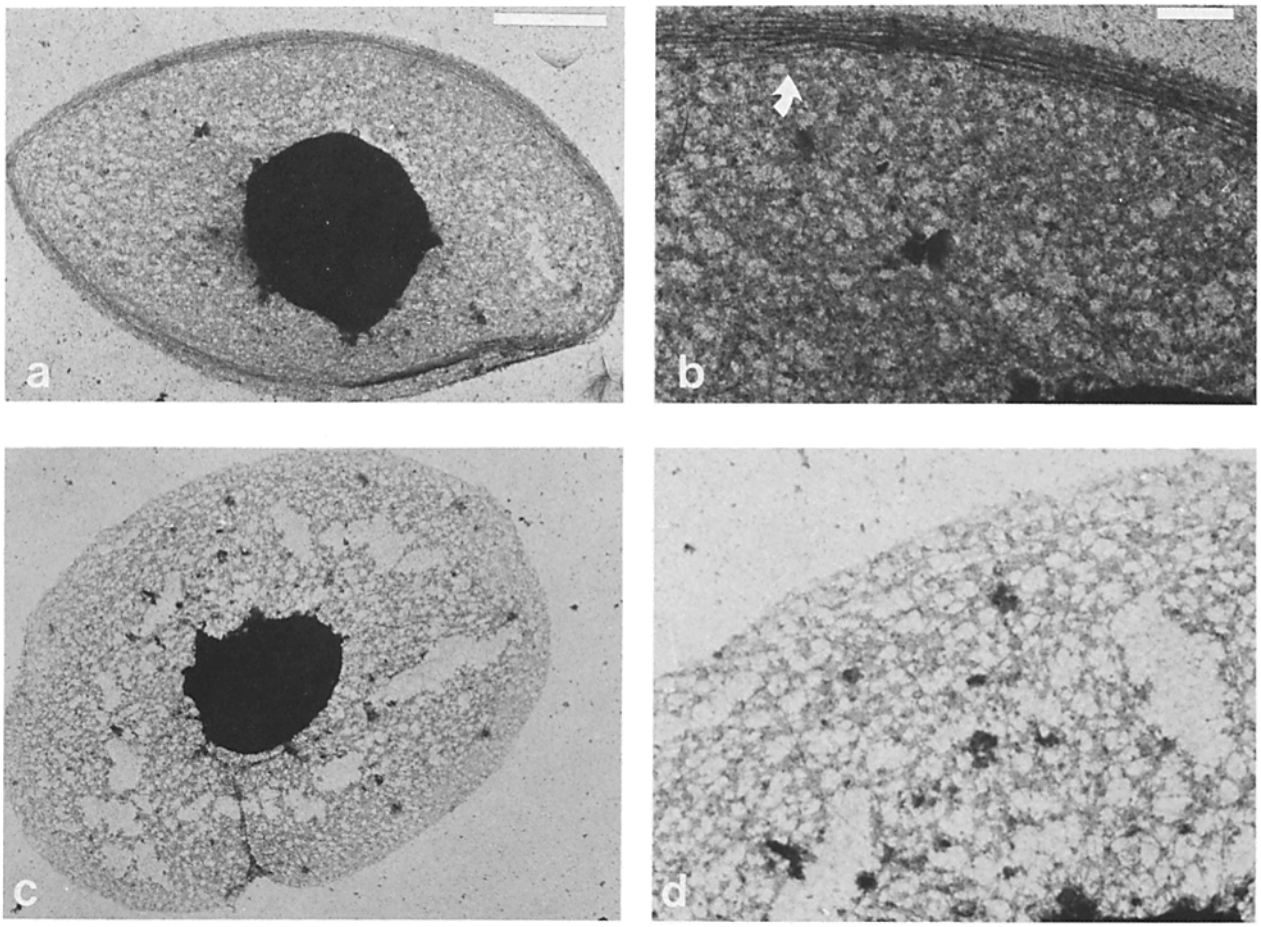

FIGURE 2 Whole mounts of detergent-extracted erythrocytes with and without microtubules. (*a*) Cell incubated in the warm for 90 min before extraction. The tips of the cell are slightly distorted. Bar, 2 μm. (× 7,000). (*b*) A 2.9-fold enlargement of the same cell. Bar, 0.5 μm. (*c*) Cell incubated in the cold for 90 min before extraction (× 7,000). (*d*) A 2.9-fold enlargement of the same cell. The enlargements shown in *b* and *d* are those used to score the number of microtubules. We count eight microtubules at the arrow in *b*.

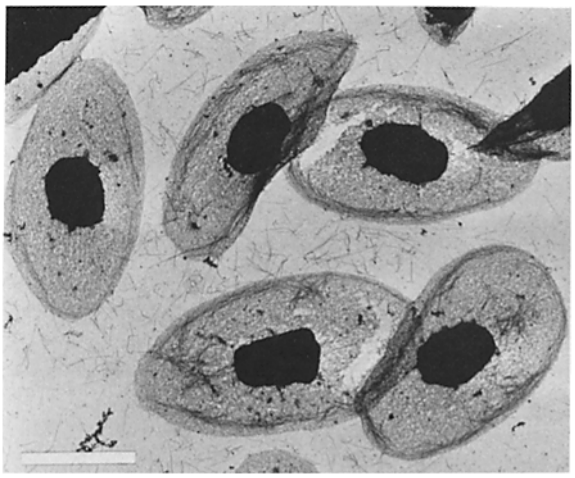

FIGURE 3 Field of whole mounts with marginal bands regrown in vitro. The experimental conditions are described in the text. The microtubules visible between the cells presumably have broken off from the cells, since no microtubules are seen if the tubulin is incubated without cytoskeletons. Extracellular microtubule fragments are also observed when cells with intact bands are extracted and fixed (12). Bar, 5 μm. × 3,000.

When microtubules are located in the cell elsewhere than at the perimeter, the cells are generally distorted. This is particularly true at the ends of the cells. We believe this distortion to arise during the washing steps the regrown cells undergo, since native cells with intact marginal bands are distorted at their ends in similar ways by this washing procedure.

The unusual, curved shape of the marginal band microtubules is also found in the structures formed in vitro. Fig. 5*a* shows a high magnification view of regrown microtubules. Note that all the microtubules have curved around the bend at the tip of the cell. Occasionally, the microtubules leave the cell. Fig. 5*b* shows a high magnification view of the opposite end of the same cell. In this instance the microtubules begin to curve, but all leave the detergent-extracted cytoskeleton and continue at a tangent. These microtubules and those which have broken off from the cells, are perfectly straight, like typical microtubules of calf-brain tubulin.

The extent of microtubule regrowth in vitro can be compared to the extent of regrowth in vivo. Fig. 6 shows a histogram of the number of microtubules in marginal bands regrown in vitro in one particular experiment. Regrowth was for 10 min; the concentration of added tubulin was 2 mg/ml. The histogram overlaps considerably with the histogram of the number of microtubules in mature erythrocyte marginal bands (12). In vitro regrowth apparently is not as efficient as in vivo regrowth: the average number of microtubules in the band is 7.2 in vitro compared to 10.6 in vivo (12). The more strenuous treatment of the regrown microtubules may have contributed to the lower values, since even native bands have

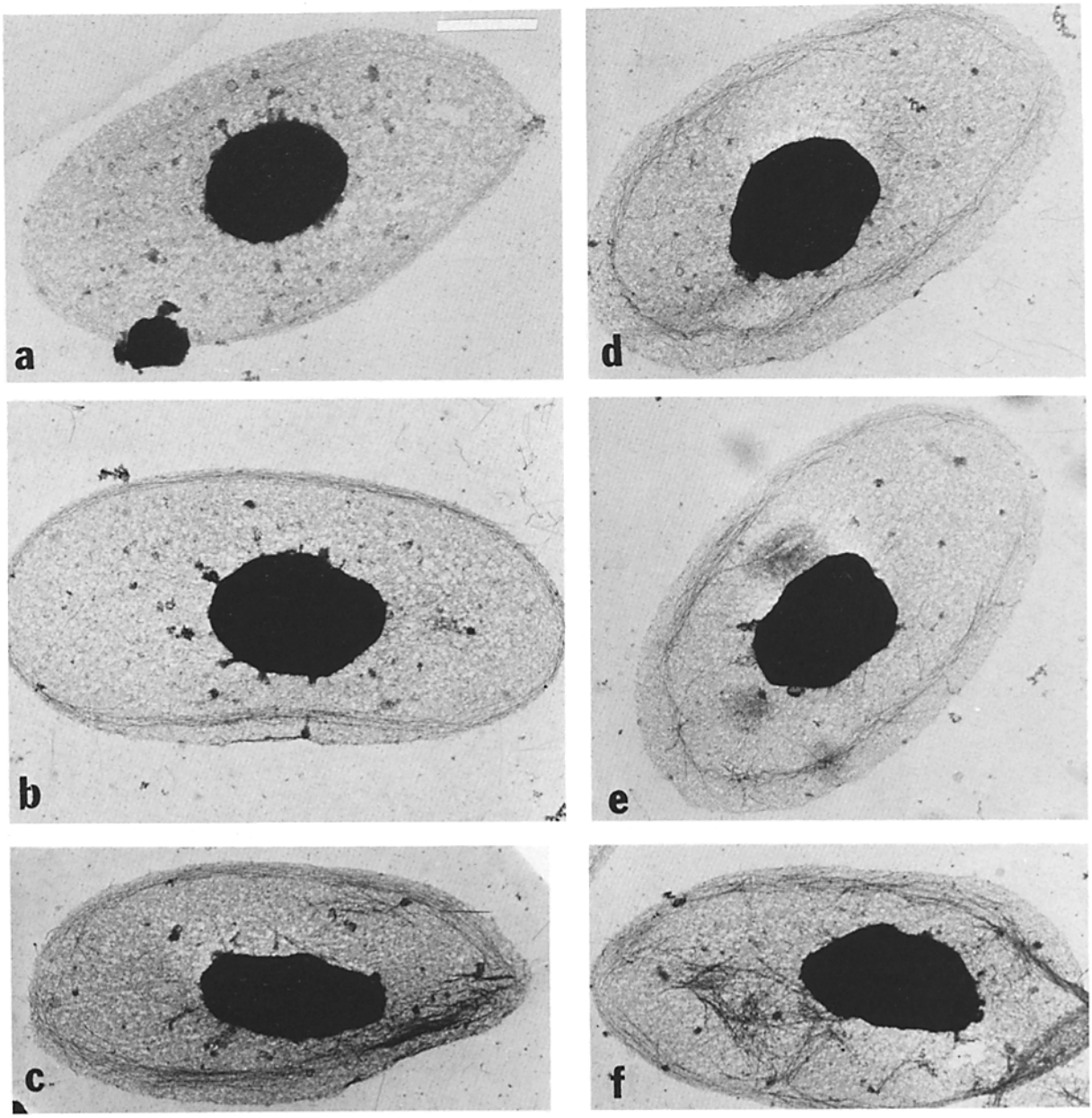

FIGURE 4 Examples of regrown marginal bands. The images are described in the text. Bar, 2 μm. × 7,000.

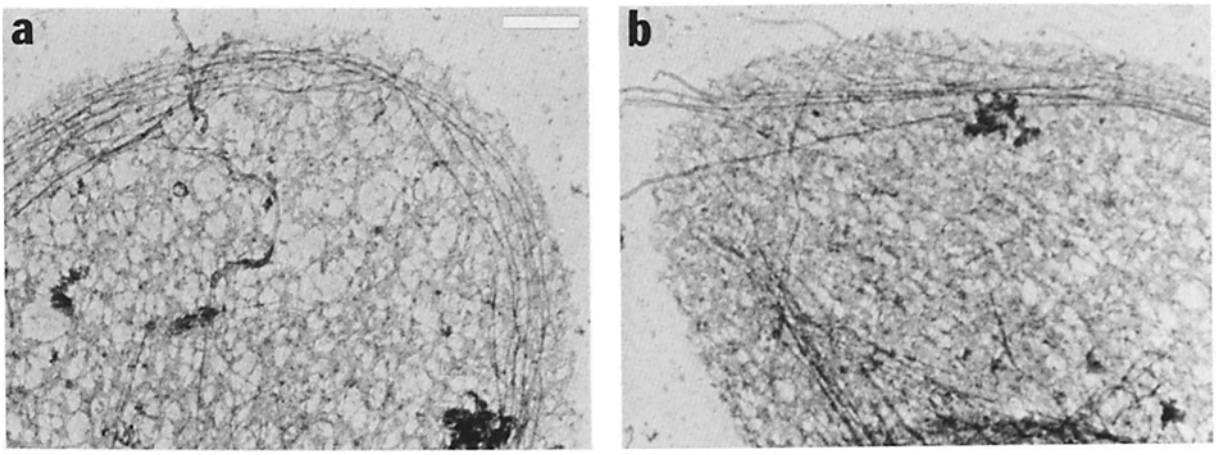

FIGURE 5 High magnification views of regrown marginal bands. (*a*) The end of a cytoskeleton where all the microtubules curve to follow the margin of the cytoskeleton. (*b*) The other end of the same cytoskeleton, showing microtubules which extend beyond the margin of the cell part way through the curve. Bar, 0.5 μm. (*a*) × 20,000, (*b*) × 19,000.

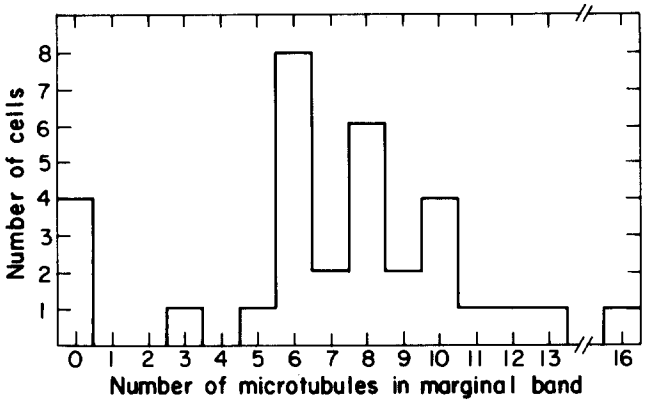

FIGURE 6 Histogram of the average number of regrown microtubules per marginal band in a typical experiment. In ~90% of the cells, the number of microtubules in the marginal band varied by plus or minus one when counted at several different points around the perimeter, as is the case with the marginal bands formed in all cells in vivo. Those marginal bands formed in vitro where the number of microtubules that varied by by two or more were excluded from the histogram.

fewer microtubule profiles when incubated and washed using this same procedure. However, the maximum number of microtubules at the periphery ever seen in whole mount preparations of a regrown band is 16, the maximum seen in vivo. We have regrown marginal bands using two to three times as much tubulin, and twice the incubation time, and have never seen more than 16 microtubules at the periphery of the cells.

DISCUSSION

The position of the marginal band of nucleated erythrocytes and the shape of the microtubules in it make the band an unusual microtubule organelle. Unlike other animal cells, the microtubules are all at the periphery where they curve to form an elliptical ribbon. In a large population of normal cells, this structure repeats in each member. These properties make the nucleated erythrocyte an attractive system for studying the mechanisms by which microtubules are organized in the cytoplasm. We show here that the major features of the marginal band—its location within the cell and the curvilinear form of its microtubules—can be faithfully recapitulated in an in vitro reconstitution system. That system combines detergent-extracted cytoskeletons of avian erythrocytes that have been depleted of microtubules with purified preparations of calf brain tubulin, which themselves are incompetent to assemble. The results show that there are components of the detergent-extracted cytoskeletons that can promote the tubulin to assemble. The resulting microtubules are not randomly located within the cell. Rather, most if not all of the microtubules are at the position of the marginal band. In addition the resulting microtubules are not the straight structures typically produced by in vitro assembly reactions. Instead, they curve as the cell curves, even managing to negotiate the rather sharp turns at the ends of the ellipse. This result demonstrates that all the components necessary for constructing a marginal band are retained in the cytoskeletons even after they have been depleted of microtubules by incubation at 4°C in vivo.

This work is based on two previous findings. First, Brinkley and colleagues (3, 11) showed that purified tubulin that was incompetent to self-assemble would in fact form microtubules in detergent-extracted cytoskeletons of 3T3 cells. Those microtubules apparently arose from MTOCs at or near the centrioles in those cells, suggesting that the MTOCs were providing a necessary nucleating function as they may do in vivo. However, the pattern of the reformed microtubules was clearly different from that of the native complex. The reformed microtubules were straight; they were directed randomly from the MTOC; and they apparently extended beyond the periphery of the cell. In vivo, the microtubules are typically wavy; they are more numerous in some regions of the cytoplasm than others; and they obviously do not extend beyond the plasma membrane. In those cells then, the MTOC and the cytoskeleton could initiate microtubule assembly but not organize the products into the in vivo microtubule pattern. In this laboratory, we have repeated this experiment with other cells, such as neuroblastoma with neurites extended, and obtained the same results: microtubules were formed but not in the appropriate pattern (M. Magendantz and F. Solomon, unpublished results).

The second basis for these experiments was a previous investigation of marginal band reformation in vivo. After cold-induced depolymerization of the microtubules, the intact cells could regenerate the marginal band in almost every detail—position, shape of the microtubules, their number and apparent length. The quite precise end-point of this reformation allowed us to treat it as an in vivo assembly reaction, examine its kinetics, and identify kinetically significant intermediates. By several methods, we were unable to detect either the presence or the participation of MTOCs. These results were consistent with the existence of peripheral determinants of microtubule shape in these cells. There is now independent evidence for such determinants in another system. Membrane fragments of *Distigma*, which have been depleted of microtubules, will bind formed structures with native orientation and organization (15). And in avian erythrocytes, "tracks" on the interior face of the cell membrane have been visualized (8); although their relationship to the marginal band is not yet clear.

The use of the in vitro experimental approach to this system has strengthened the conclusion that peripheral determinants are involved in marginal band formation. There is no apparent unique point of initiation of microtubules in the regrown cells. In fact, the significant proportion (40%) of cells with short microtubules at the periphery suggests that in these regrown cells there are multiple sites of microtubule initiation in vitro. Another possibility is that the final pattern we see—after regrowth, washing, fixation, and all subsequent manipulations—is the result of selective stabilization of microtubules rather than the result of the mode of nucleation.

This experiment directly addresses the role of erythrocyte specific tubulin in marginal band formation. Murphy and Wallis (14) have shown that the beta-tubulin in avian erythrocytes differs in isoelectric point and peptide map from the beta-tubulin found in avian brain. Since the microtubules in erythrocytes also differ from the microtubules found in any other cell, the two phenomena might have been related. We have unambiguously demonstrated, however, that neither erythrocyte beta-tubulin, nor even avian tubulin, is required for the formation of marginal band microtubules.

Two other features of the marginal band, the number of profiles in the band and the apparent length of the microtubules, are approached by this experiment with rather less resolution. The number of microtubules formed in vitro is

only slightly less on average that those present in mature erythrocytes in vivo (Fig. 6). More importantly, even at long times and high tubulin concentrations we do not find greater than 16 microtubules in the region of the band. However, we have refrained from concluding that the determinants are saturated, and, therefore, can themselves specify microtubule number. The in vitro reaction is not well-behaved at high concentrations of tubulin, and microtubules can be detected at unusual places—between the nucleus and the cell margin, or between cells. At high concentrations we may be detecting self-assembly. These extraneous microtubules in some cases obscure the band and complicate the interpretation of the data. Thus, the results suggest but do not prove that the number of microtubules in the band is limited by the cell. The regrown microtubules vary in apparent length over a wide range, but the chance for this result to be muddled by artifacts is great.

Our data suggest that elements of the detergent-extracted cytoskeleton can promote microtubule assembly from purified tubulin, and specify the organization of those structures. Several mechanisms of action are possible. At one extreme, it is possible that a single microtubule serves as a pioneer and that all susbequent microtubules follow it. Such a pathway relies upon interactions between microtubules, the existence of which was suggested by the isolation of intact marginal bands from newt and dogfish erythrocytes (4, 5) and from human platelets (16). However, we are adding back only tubulin in our experiments, so there are essentially no associated proteins to mediate such interactions; instead, the microtubules would have to be connected by tubulin-tubulin interconnections. Alternatively, there may be independent attachment pathways for each microtubule reformed, and each microtubule interacts with the cytoskeleton only. In this view, each microtubule is equivalent to any other, and makes the same interactions. If indeed the number of regrown microtubules is specified by the cell, as our data suggest, the model of independent microtubules is supported. We are pursuing this issue.

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