The Transport Properties of Axonal Microtubules Establish Their Polarity Orientation

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Abstract. It is well established that axonal microtubules (MTs) are uniformly oriented with their plus ends distal to the neuronal cell body (Heidemann, S. R., J. M. Landers, and M. A. Hamborg. 1981. *J. Cell Biol.* 91:661–665). However, the mechanisms by which these MTs achieve their uniform polarity orientation are unknown. Current models for axon growth differ with regard to the contributions of MT assembly and transport to the organization and elaboration of the axonal MT array. Do the transport properties or assembly properties of axonal MTs determine their polarity orientation? To distinguish between these possibilities, we wished to study the initiation and outgrowth of axons under conditions that would arrest MT assembly while maintaining substantial levels of preexisting polymer in the cell body that could still be transported into the axon. We found that we could accomplish this by culturing rat sympathetic neurons in the presence of nanomolar levels of vinblastine. In concentrations of the drug up to and including 100 nM, the neurons actively extend axons. The vinblastine-axons are shorter than control axons, but clearly contain MTs. To quantify the effects of the drug on MT mass, we compared the levels of polymer throughout the cell bodies and axons of neurons cultured overnight in the presence of 0, 16, and 50 nM vinblastine with the levels of MT polymer in freshly plated neurons before axon outgrowth. Without drug, the total levels of polymer increase by roughly twofold. At 16 nM vinblastine, the levels of polymer are roughly equal to the levels in freshly plated neurons, while at 50 nM, the levels of polymer are reduced by about half this amount. Thus, 16 nM vinblastine acts as a "kinetic stabilizer" of MTs, while 50 nM results in some net MT disassembly. At both drug concentrations, there is a progressive increase in the levels of MT polymer in the axons as they grow, and a corresponding depletion of polymer from the cell body. These results indicate that highly efficient mechanisms exist in the neuron to transport preassembled MTs from the cell body into the axon. These mechanisms are active even at the expense of the cell body, and even under conditions that promote some MT disassembly in the neuron. MT polarity analyses indicate that the MTs within the vinblastine-axons, like those in control axons, are uniformly plus-end-distal. These results indicate that MTs translocate from the cell body into the axon exclusively with their plus ends leading, and that this process is unrelated to the assembly properties of the MTs. Based on these results, we conclude that MT transport is a key component of axon growth, and that the transport properties of the MTs establish their polarity orientation.

Microtubules (MTs)[1] in the axon are uniformly oriented with respect to their polarity, with the plus ends of the MTs distal to the neuronal cell body (Heidemann et al., 1981; Burton and Paige, 1981; Baas et al., 1987, 1988, 1989, 1991*b*; Baas and Ahmad, 1992). Because the plus ends of axonal MTs are the exclusive sites from which they elongate (Okabe and Hirokawa, 1988; Baas and Ahmad, 1992), this organization has important implications for the manner by which the MT array of the axon is elaborated. In addition, because different organelles appear to be preferentially translocated toward either the plus or the minus end of the MT, the polarity orientation of MTs in the axon will, in part, determine its cytoplasmic composition (Baas et al., 1988; Black and Baas, 1989). Surprisingly, very little is known about the mechanisms by which MT organization in the axon is established. In nonneuronal cells, MT arrays of uniform polarity orientation arise via direct continuity of the minus ends of the MTs with a discrete MT nucleating structure such as the centrosome (for review see Brinkley, 1985). In contrast, axonal MTs are not attached to any such nucleating structure (Lyser, 1968; Sharp et al., 1982), but rather stop and start at multiple sites along the length of the axon (Bray and Bunge, 1981; Tsukita and Ishikawa, 1981). In the absence of continuity with a centrosome-like nucleating structure, how is the distinctive polarity orientation of axonal MTs established?

Three possibilities seem worthy of consideration. The

1. *Abbreviations used in this paper*: DIC, differential-interference contrast; MAP, microtubule-associated protein; MT, microtubule.

polarity orientation of axonal MTs may be a consequence of their transport properties, their assembly properties, or the MT bundling properties of accessory proteins. At present, the latter seems unlikely. Expression of tau, an axon-enriched microtubule-associated protein (MAP), in normally rounded Sf9 cells causes them to extend processes containing predominantly plus-end-distal MTs (Baas et al., 1991*a*). However, a similar response is obtained when at least one other MAP that is not axon specific is expressed in these cells (Le Clerc et al., 1993), suggesting that factors other than MAP composition probably regulate MT polarity orientation. Moreover, it is unclear how the bundling properties of MAPs could ensure that MT bundles of common polarity orientation are always oriented with plus ends and not minus ends distal to the cell body. In light of these considerations, attention shifts to either MT assembly or MT transport as the factor which determines the polarity orientation of MTs in the axon.

At present, little information is available concerning the relative contributions of MT transport and assembly to the organization and elaboration of the axonal MT array. In fact, the issue has been a matter of some controversy, with certain authors disclaiming any contribution of MT transport whatsoever. The controversy originally stemmed from studies in which anti-MT drugs were applied to localized regions of the axon (Bamburg et al., 1986). Axons ceased growing when the drugs were applied to the axon tip, but continued growing when the drugs were applied to the proximal region of the axon. The authors equated MT transport with the addition of tubulin subunits onto the minus ends of MTs in the proximal region of the axon. Therefore, when axons continued to grow after drugs were applied to this region, the authors concluded that MT transport is not required for axon growth. Since then, several authors have argued that MT transport is inconsistent with the local MT assembly thought to occur at the distal tip of the axon (see for example Gordon-Weeks, 1991). These interpretations are problematic in that they confuse the transport properties of MTs with their assembly properties. MT transport, by definition, is the movement of a MT from one location to another. This can occur irrespective of whether the MT is elongating, not undergoing a length change, or even shortening.

Several distinct lines of evidence strongly favor the view that MT transport is an important feature of axon growth. Perhaps the most compelling is the inarguable need for an active transport mechanism to move tubulin from its site of synthesis within the cell body to distal sites in longer axons. In fact, the movement of tubulin from the cell body down the axon has been extensively studied for over a decade, and the kinetics clearly indicate an active transport mechanism (for review see Lasek, 1982, 1986, 1988). In addition, Black et al. (1986) have reported that a large fraction of tubulin becomes incorporated into an insoluble form, presumably polymer, very rapidly after its synthesis in the cell body. Consistent with these findings, three recent lines of evidence from our laboratory strongly support a cell body origin for axonal MTs. First, the results of nocodazole-recovery experiments indicate that all MT assembly in the axon occurs via the elongation of existing polymer (Baas and Ahmad, 1992; see also Baas and Heidemann, 1986). This finding suggests that the entirely new MTs required for axon growth do not arise within the axon itself, and hence implicates the cell body as the source of new MTs for the growing axon. Consistent with this idea, we have determined that gamma-tubulin, a newly discovered protein shown to be essential for in vivo MT nucleation in nonneuronal cells (Joshi et al., 1992), is restricted to the centrosome in neurons (Baas and Joshi, 1992). If gamma-tubulin is also required for MT nucleation in the neuron, then this observation supports the idea that the MTs destined for the axon are first nucleated at the centrosome, and then released for transport into the axon. Finally, we have shown that the majority of the stable MT polymer for the axon is generated in its most proximal region, suggesting that much of the stable polymer throughout the axon arises proximally, and is then translocated to more distal sites (Baas et al., 1993).

Despite all of these observations, the existence of MT transport in the axon remains controversial because of mixed results obtained from photoactivation and/or photobleach studies on the behavior of MTs in the axon. The essence of this approach is to put a narrow mark across the MT array of the axon, and then monitor for movement of the marked polymer. Some of these studies have failed to show movement of the marked MTs (Lim et al., 1989, 1990; Okabe and Hirokawa, 1989, 1992), while other studies have shown proximodistal movement (Keith, 1987; Reinsch et al., 1991; Okabe and Hirokawa, 1992). The reasons for these differing results are unknown. One possibility is that the axons of different kinds of animals are differentially sensitive to the potential for photodamage inherent in the technique. Whatever the reason, it is clear that new approaches are needed to study MT transport in the axon.

To study MT transport in the axon, we have sought a method to dissect apart the contribution of MT transport from that of MT assembly in elaborating the axonal MT array. To do this, we have taken advantage of recently discovered properties of vinblastine. Vinblastine is an older MT drug, with newly discovered properties. When applied to cells in millimolar concentrations, the drug induces the formation of tubulin paracrystals. However, when used in the nanomolar range, the drug does not induce paracrystals. Instead, it either induces low levels of MT depolymerization, or actually arrests MT assembly without depolymerizing existing MTs, thus acting as a "kinetic stabilizer" of MTs (Jordan and Wilson, 1990; Jordan et al., 1991, 1992). In the present study, we report that when neurons are cultured in the presence of nanomolar levels of vinblastine, they grow axons. These axons are shorter than control axons, but nevertheless contain MTs. Moreover, there is a progressive increase in MT polymer in the axons as they grow, and a corresponding decrease in polymer from the cell body. These results indicate that highly efficient mechanisms exist in the neuron to transport preassembled MTs from the cell body into the axon. To determine whether these transport mechanisms, separate from the assembly properties of the MTs, can account for the polarity orientation of axonal MTs, we determined the polarity orientation of MTs in axons grown in the presence of nanomolar levels of vinblastine.

Materials and Methods

Cell Culture

Sympathetic neurons from the superior cervical ganglia of newborn rat pups were cultured either as dissociated cells or as explants. For cultures of dissociated cells, the ganglia were treated with 0.25 mg/ml collagenase for 1 h followed by 0.25 mg/ml trypsin for 45 min, and then triturated with a pasteur pipet into a single cell dispersion. The neurons were then plated onto "special dishes" that were prepared by adhering a glass coverslip to the bottom of a 35-mm plastic petri dish into which had been drilled a 1-mm-diam hole (Whitlon and Baas, 1992; Ahmad et al., 1993). Before plating the cells, the glass-bottomed well of the special dish was treated for 3 h with 1 μg/ml polylysine, rinsed extensively, and then treated with 10 μg/ml laminin for 4 h (Higgins et al., 1991). Cells were plated in media consisting of Leibovitz' L-15 (Sigma Chem. Co., St. Louis, MO) supplemented with 0.6% glucose, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% FBS (Hyclone Labs., Logan, UT), and 100 μg/ml NGF. For explants cultures, ganglia were cut into three pieces and plated onto collagen-coated plastic petri dishes as previously described (Baas and Black, 1990; Baas and Ahmad, 1992).

Vinblastine Treatment

Vinblastine sulfate was purchased from Sigma Chem. Co., and dissolved in HPLC grade methanol (Aldrich Chem. Co., Milwaukee, WI) at a concentration of 4 mM. This stock was further diluted with tissue culture medium to concentrations of 4 μm and 0.4 μm. To make the final culture media for plating cells, we used the most concentrated of these three stocks from which we could accurately measure the appropriate amount of vinblastine. Media were prepared that contained 0, 4, 16, 50, 100, and 500 nM vinblastine. The vinblastine had no deleterious effects on cell attachment, so neurons were plated directly into the drugged media. The cultures were generally grown overnight (20 h) before preparation for microscopic analyses. In one experiment, the longer-term effects of the drug were analyzed. For this experiment, the media in some cultures were exchanged with fresh drugged media the following morning, while in other cases, the plating media were not changed. In all cases, both the vinblastine-containing media and the vinblastine stock solutions were prepared fresh, and were insulated from light as much as possible.

Transmission Electron Microscopy

Cultures were prepared for transmission electron microscopy by conventional means. Briefly, cultures were fixed by replacing the media with 0.1 M cacodylate (pH 7.0) containing 2% glutaraldehyde and 2 mg/ml tannic acid. After 15-20 min, the cultures were rinsed two times for 5 min each in 0.1 M cacodylate, postfixed for 15 min in 0.1% osmium tetroxide, rinsed twice for 2 min each in 3.6% NaCl, rinsed twice for 2 min each in water, stained for 1 h in the dark with 5% uranyl acetate, dehydrated in ethanols, and embedded in LX-112 (Ladd Res. Inds., Inc., Burlington, VA). The pre-embedment uranyl acetate treatment improved the contrast of our samples, permitting easier identification of MTs (see Banker and Goslin, 1991).

Morphometric Analyses

10 control neurons and 10 each of neurons plated in the presence of 4, 16, 50, and 100 nM vinblastine were analyzed morphometrically as follows. Lengths and widths of axons were obtained by direct measurements of cells visualized with differential-interference contrast (DIC) microscopy using a 35M Axiovert microscope with a 40× Planneofluor objective (Carl Zeiss, Inc., Thornwood, NY). Based on electron microscopic analyses of the neurons, axons were assumed to be slightly flattened cylinders with actual diameters somewhat shorter than the width of the axon measured under DIC optics, and cell bodies were assumed to be slightly flattened spheres with actual radii somewhat shorter than the average distance from cell center to periphery. The cell bodies and axons were both found to become progressively flatter with increasing vinblastine concentration, and based on electron microscopic analyses, fudge-factors were obtained to adjust the measured widths and radii accordingly. These factors were 1.00, 0.75, 0.75, 0.70, 0.65, and 0.55 for freshly plated neurons (nearly spherical), control overnight neurons, and neurons plated in 4, 16, 50, and 100 nM vinblastine, respectively. Estimates for the volume of cytoplasm in the cell body and axonal arbor for each cell were obtained using these adjusted values and the formulas for the volumes of a sphere and cylinder, respectively. The cytoplasm from all of the axons of a single neuron were summed. An estimate for the volume of the nucleus was obtained in a similar manner as that for the cell body, and this value was subtracted from the total volume calculated for each cell body. Numbers of axons emerging from each cell body, degree of branching, and frequency of swellings along the lengths of the axons were also analyzed. Values were recorded directly on video-prints obtained using a video graphic printer (model UP-870MD; Sony Corporation, Japan), and subsequent ultrastructural analyses were performed on the same cells from which these morphologic data were obtained.

Standard transmission electron microscopy was used to quantify the levels of MT polymer in the cell bodies of freshly plated control neurons (which had not yet grown axons), in the cell bodies and axonal arbors of control neurons that were permitted to grow axons overnight, and in the cell bodies and axonal arbors of neurons that had been plated in the presence of various concentrations of vinblastine. For each condition, three randomly selected neurons among the 10 analyzed morphometrically under DIC optics were analyzed ultrastructurally. For these studies, neurons were thin sectioned completely or nearly completely, and picked up on standard 200 mesh copper grids. Cell bodies and axons appearing on the grids were then photographed, but no special effort was made to ensure that 100% of each cell was photographed. Rather, the quantitative morphology obtained at the light microscopic levels was used to obtain an estimate for the proportion of the total volume encompassed within our electron micrographs, and this proportion was used to obtain estimates for the total volumes and MT mass obtained within the cell body and axonal arbor of each neuron. For each neuron, at least 25% of the total cytoplasm of the cell body and 25% of the total cytoplasm in the axonal arbor were analyzed.

In other studies designed to determine the lengths of individual MTs in the axons of control and experimental neurons, the MT arrays of control and vinblastine-axons were serially reconstructed by our previously described method (Baas and Heidemann, 1986; Joshi et al., 1986). Briefly, serial sections of individual axons were obtained using an Ultracut E Ultramicrotome (Reichert Jung, Vienna), collected on formvar-coated slot grids, and photographed with a CX-100 electron microscope (JEOL USA Inc., Peabody, MA). Membranous borders and MTs were traced from the electron micrographs onto transparent plastic sheets. The tracings were aligned first using the membranous borders as registration markers, and were then aligned to maximize the matches of MT ends in consecutive sections by moving the tracings within a range of two MT diameters. MT lengths and organization were then depicted in the form of composite drawings.

Microtubule Polarity Determinations

To determine the polarity orientation of axonal MTs in control and experimental neuron cultures, we used the standard "hooking" protocol (Heidemann and McIntosh, 1980; Baas et al., 1989; for review see Heidemann, 1991). In this procedure, cultures are lysed in the presence of a special MT assembly buffer containing exogenous brain tubulin, and then prepared for electron microscopy by conventional means. The exogenous tubulin adds onto existing MTs in the form of lateral protofilament sheets that appear as "hooks" on the MTs when viewed in cross section. The curvature of the hook reveals the polarity orientation of the MT; a clockwise hook indicates that the plus end of the MT is directed toward the observer, while a counterclockwise hook indicates the opposite. In the present study, control and experimental explant cultures were rinsed twice in PBS (to remove tissue culture medium and residual vinblastine that might interfere with hooking), and then treated for 30 min at 37°C with 0.06% Brij 58 in a MT assembly buffer (0.5 M Pipes, 0.1 mM EGTA, 0.01 mM EDTA, 0.1 mM $MgCl_2$, 2.5% DMSO, 0.5 mM GTP) containing 1.2 mg/ml MT protein. Explant cultures were used for these experiments because they maximize the number of aligned axons that can be cross sectioned simultaneously. After hooking, the cultures were fixed by the addition of an equal quantity of 4% glutaraldehyde, and prepared for electron microscopy. Cross sections of the axons were then taken, and hooks were interpreted and scored as previously described (Heidemann and McIntosh, 1980; Heidemann, 1991). As in our previous work, hooks were judged to be clockwise or counterclockwise from the vantage point of the distal tip of the axons.

Results

Neurons Cultured in Vinblastine Extend Axons

Our first goal was to define culture conditions under which neurons could be plated that would arrest MT assembly without substantially depolymerizing the existing MTs. In this way, we could determine whether preexisting MTs are transported into a growing axon, independent of new MT assembly. In previous studies, it was determined that 4 nM vinblastine kinetically stabilizes MTs in vitro and in nonneuronal cells, arresting MT assembly without inducing detectable MT disassembly (Jordan and Wilson, 1990; Jordan et al., 1991, 1992). Because the precise concentration at which vinblastine kinetically stabilizes MTs is likely to vary among different cell types, it was necessary for us to investigate the effects of various concentrations of the drug on neurons. Rat sympathetic neurons were cultured in media containing vinblastine sulfate at concentrations of 0, 4, 16, 50, 100, and 500 nM. In the presence of these concentrations, the neurons attached to the substratum within the first 30 min of plating and began extending lamellipodia, entirely similar to the controls. The following morning, 20 h after plating, cultures were examined with DIC and phase-contrast optics, and photographed. In cultures plated in all drug concentrations except 500 nM, virtually all of the neurons extended axons overnight. At 500 nM, <10% of the cells had axons, and these were generally <10 μm in length.

In a limited number of experiments, we examined the longer-term effects of vinblastine on the cultures. If the concentrations of vinblastine that we are using are sufficient to permanently arrest MT assembly, we would expect the neurons to die shortly after their overnight bout of axon outgrowth. Indeed, by the second day in culture, all of the neurons plated in 500 nM vinblastine, and the vast majority of the neurons plated in 50 and 100 nM had died. The few living cells remaining had undergone no further axon growth since the previous day, and these cells died by the third day. At 4 and 16 nM, no cell death was apparent by the second or third day, and the axons continued to grow longer, but at a slower rate than controls. In addition, dendritic development occurred at these lower drug concentrations, similar to controls, suggesting that most or all metabolic processes in the cells are still active. However, consistent with the previous findings on nonneuronal cells (Jordan et al., 1991, 1992), virtually all of the nonneuronal cells that typically contaminate our cultures died by the second day, even at 4 and 16 nM. One possibility is that, unlike the nonneuronal cells, the neurons may be able to metabolize low levels of the drug over time. However, arguing against this possibility, entirely similar results were obtained if the cultures were fed daily with fresh vinblastine-containing medium. Other possibilities are that neurons may be able to compensate for the presence of drug by synthesizing higher levels of tubulin, or simply that these results reflect the greater stability of neuronal MTs relative to the MTs in nonneuronal cells (see for example Baas and Black, 1990). Whatever the reason, neurons and nonneuronal cells are clearly different with regard to their sensitivity to vinblastine, suggesting that higher concentrations of the drug are probably required to arrest MT assembly in neurons, at least over prolonged periods of time.

All subsequent efforts were focused on the overnight cultures. Before directly quantifying the effects of the drug on MT mass in the neurons, we further characterized the levels of axon outgrowth and morphologic features of the cultures. DIC images of neurons grown in the presence of 0, 16, 50, and 100 nM vinblastine for 20 h are shown in Fig. 1, data on the average volume of cytoplasm within cell bodies and axonal arbors of neurons are shown in Fig. 2, and our system for quantifying cytoplasmic volume is described in Materials and Methods. In freshly plated cultures, there are no axons, and the average volume of cytoplasm in the cell body is ≈900 μm^3. In control overnight cultures, there is an increase in the cytoplasmic volume of the cell body to ≈1,150 μm^3, and ≈750 μm^3 of axoplasm are elaborated, resulting in a total increase in volume of roughly double (Fig. 1 *a*). At 4 nM, the cultures are generally similar in appearance to overnight controls, except that the cell body volume is slightly less, and the volume of axoplasm is roughly half. Compared with freshly plated neurons, the overall cytoplasmic volume at 4 nM increases by ≈40%. At 16, 50, and 100 nM (shown in Fig. 1, *b*, *c*, and *d*, respectively), the volume of the cell body decreases slightly relative to freshly plated neurons, and the volume of axoplasm is ≈9% of that in control overnight neurons, resulting in little change in total cell volume compared with freshly plated neurons. In addition, we consistently noticed that, at all concentrations of vinblastine above 4 nM, there appeared to be an inverse relationship between the size of the cell body and the size of the axonal arbor extended by an individual neuron. That is, smaller cell bodies generally accompanied larger axonal arbors and vice versa, suggesting that the cell body is losing volume at the expense of the axons at vinblastine concentrations higher than 4 nM.

The principal difference among neurons grown in 16, 50, and 100 nM vinblastine is not in cell body or axoplasmic volume, but in morphologic features of the axonal arbor, such as branching and beading. At 16 nM, the axons are generally healthy in appearance, with no greater frequency of branching or beading than axons in control overnight or 4 nM cultures. At 50 and 100 nM, the axon arbors are collectively similar in overall size to the arbors at 16 nM, but the arbors are far more extensively branched, and a greater number of axons emerge from a single cell body (compare Fig. 1 *b* with Fig. 1, *c* and *d*). In addition, there is a far greater frequency of beads along the lengths of the axons at 50 nM compared with 16 nM, and a greater frequency yet at 100 nM. Previous work indicates that the frequency of cytoplasmic swellings or "beads" increases along the length of the axon with decreasing MT mass (Horie et al., 1983; Joshi et al., 1986), and the number of branch points decreases with increasing MT mass (Letourneau et al., 1986). Thus, the present findings are consistent with the expectation that cultured neurons will contain progressively fewer and shorter MTs when plated in increasing concentrations of vinblastine.

Effects of Vinblastine on Microtubule Polymer Levels in the Neuron

To determine whether the vinblastine-axons contain MTs, we used standard transmission electron microscopy. This method permits the unequivocal identification of MTs, as well as information on their organization. Electron micrographs indicate that MTs are present in the axons (as well as cell bodies) of neurons plated in all concentrations of vinblastine analyzed (4, 16, 50, and 100 nM). As expected, no tubulin paracrystals were found at these relatively low drug concentrations, but were detected at 500 nM, the concentration at which axon outgrowth is virtually abolished. If any of the vinblastine concentrations lower than 500 nM completely arrests MT assembly, then the MTs present in the axons would have to have arrived by transport from the cell body. To test this, we quantified the levels of MT polymer present within the cell bodies of freshly plated neurons (which had not yet grown axons), in the cell bodies and ax-

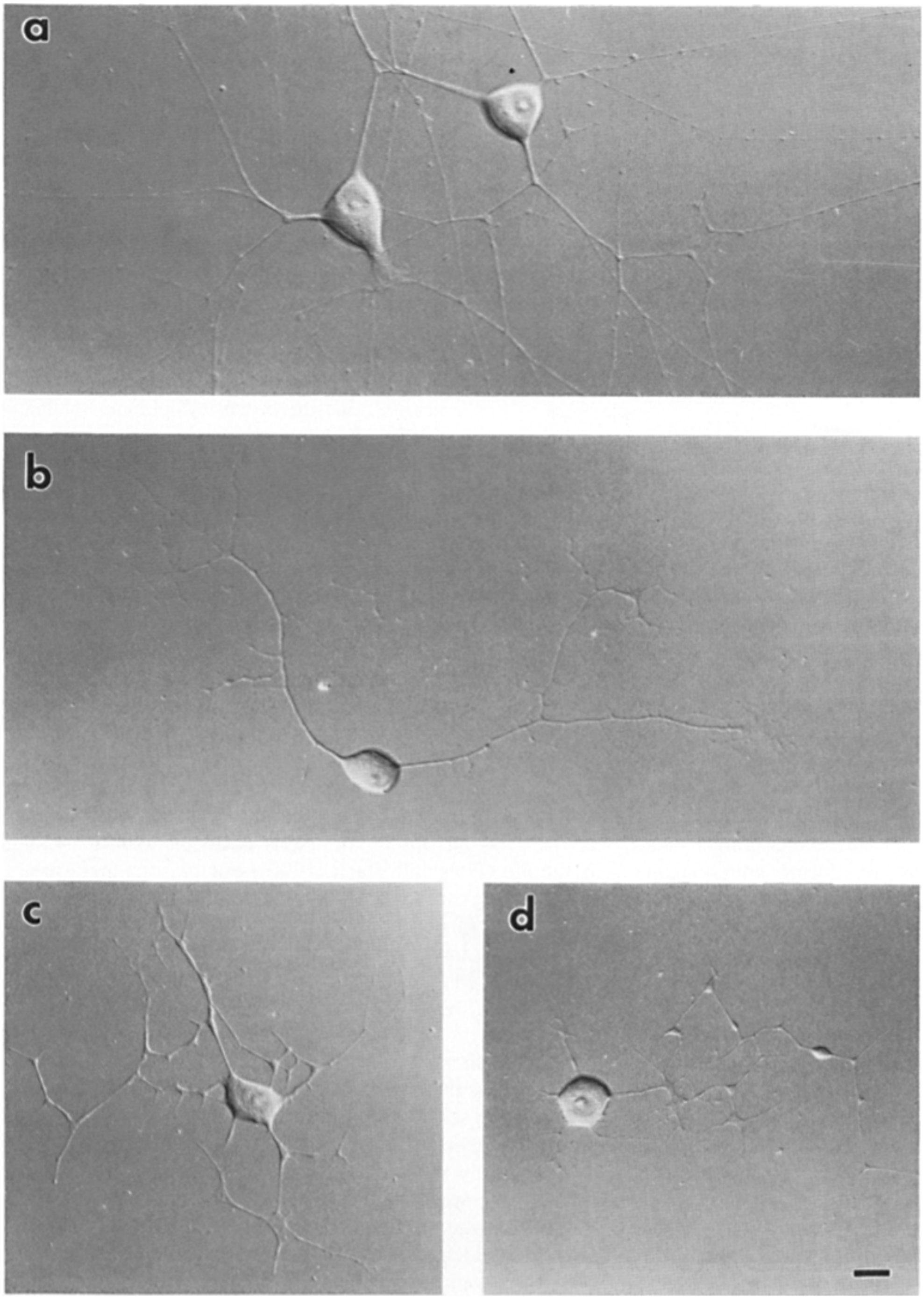

Figure 1. DIC micrographs of rat sympathetic neurons cultured in the presence of 0, 16, 50, and 100 nM vinblastine for 20 h in *a–d*, respectively. In the control cultures, axonal outgrowth is extensive, with the axons from neighboring neurons forming a dense network with one another. In the presence of all three drug concentrations, axonal outgrowth is apparent, but far less elaborate than in controls. The volume of cytoplasm in both cell bodies and axonal arbors is lower in the neurons plated in vinblastine than in control neurons. With increasing concentrations of vinblastine, there is an increase in the frequency of branching, and beading along the length of the axon. In addition, in the presence of vinblastine, the cytoplasmic volume of the cell body appears to progressively decrease as the volume of the axonal arbor increases. See Results and Fig. 2 for more details and quantitative information. Bar, 10 μm.

onal arbors of control neurons that were permitted to grow axons overnight, and in the cell bodies and axonal arbors of neurons that had been plated in the presence of vinblastine. For the latter, we focused on the 16 and 50 nM concentrations because all other indications suggest that 4 nM is probably insufficient to arrest MT assembly in these cells, and 100 nM is approaching the concentration which induces paracrystals. The methods for quantification of MTs are described in Materials and Methods, examples of electron micrographs are shown in Fig. 3, and the data are depicted graphically in Fig. 4.

The cytoplasm of freshly plated neurons is dense in appearance, and contains many MTs (Fig. 3 *a*). This appearance is very different from the cell bodies of control overnight cultures (Fig. 3 *b*), which have a less dense cytoplasm and less than half the MT mass (≈8,000 μm compared with ≈20,000 μm). This appearance is remarkably similar to that of amputated collapsed axons (Baas and Heidemann, 1986), and probably relates to the fact that freshly plated neurons have recently retracted and incorporated into their cytoplasm some portion of their MT-rich axons, which were severed during dissection, enzymatic digestion, and trituration. In overnight control cultures, there are ≈36,000 μm of total MT polymer in the axonal arbor. These results, coupled with the diminution of MT polymer from the cell body during the overnight bout of axon outgrowth, suggest that at least 12,000

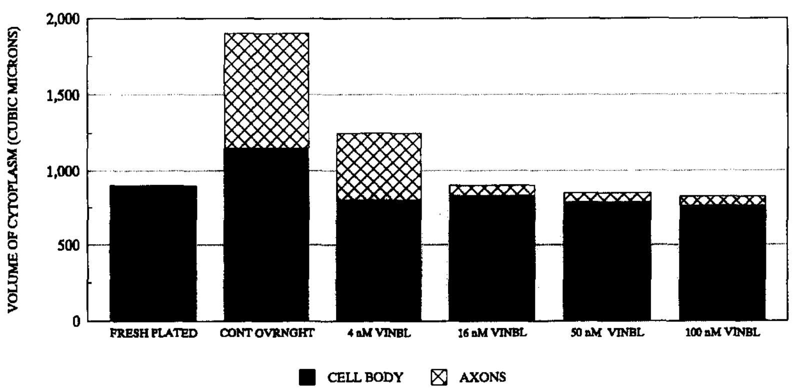

Figure 2. Graph showing the volume of cytoplasm in the cell bodies and axonal arbors of freshly plated neurons (with no axons), and neurons plated in the presence of 0, 4, 16, 50, and 100 nM vinblastine for 20 h (i.e., overnight). Volumes were calculated as described in Materials and Methods. The cytoplasmic volume of the cell body is shown in black, and axoplasmic volume is shown in hatches. There is an increase in cytoplasmic volume in the cell body as well as in the newly grown axonal arbor after 20 h with no drug. At 4 nM vinblastine, the overall cytoplasmic volume increases above that of freshly plated neurons, but is substantially less than in control overnight cultures. At all other vinblastine concentrations, the axoplasmic volume is reduced even more relative to control overnight neurons, and the overall cytoplasmic volume is roughly the same as in freshly plated neurons.

μm of MT polymer in the axons may have been delivered into the axon directly by transport from the cell body (and this very conservative estimate assumes that all of the rest of the polymer increase is due to local elongation of polymer within the axon itself). At 16 nM vinblastine, the total levels of polymer in the cell body and axonal arbor are both about half their respective values in the control overnight cultures. Very importantly, the total volume of MT polymer at 16 nM is indistinguishable from that at the time of plating. This suggests that a 16-nM concentration of vinblastine, at least during this overnight exposure, acts as a kinetic stabilizer of MTs, arresting MT assembly without inducing detectable MT disassembly. (We suspect that this is not true over longer incubation times in 16 nM vinblastine, during which axons continue to grow and dendrites begin to develop; see above.) At 50 nM vinblastine, the levels of polymer in the cell body and axonal arbor have both been further reduced by about half compared to 16 nM, indicating that 50 nM vinblastine induces net MT disassembly in cultured neurons. Qualitative observations suggest that the MT mass at 100 nM vinblastine is even further diminished (data not shown). Collectively, these results demonstrate that, under conditions which arrest MT assembly, and even promote fairly substantial MT disassembly, MTs are still present in the axons of these neurons during their growth. The simplest interpretation of these findings is that MTs within the cell body have translocated into the axons during their growth. If this is correct, the transport properties of neuronal MTs are highly potent, delivering MTs into the axon even at the expense of a cell body that cannot replenish itself with new MTs.

An alternate possibility is that MTs are able to assemble into the growing axon in the presence of vinblastine, but that the net polymer levels in the neuron remain the same or decrease because of substantial MT disassembly in the cell body. This possibility is remote, and would not be predicted by previous work on the actions of vinblastine or other anti-MT drugs, nor by previous work on MT dynamics in living cells. Nevertheless, we took a two-pronged approach to explore this formal possibility. Our initial strategy was to try to distinguish newly assembled MT polymer from preassembled polymer by differences in their staining for post-translationally modified α-tubulins in immunofluorescence and immunoelectron microscopic assays. Detyrosination and acetylation are polymer-specific and time-dependent modifications of α-tubulin that accumulate with the age of the polymer, thus rendering older polymer richer in acetylated α-tubulin and newly assembled polymer richer in tyrosinated α-tubulin (see Baas and Black, 1990; Baas et al., 1991*b*; Baas and Ahmad, 1992). In neurons grown in the presence of vinblastine, the levels of tyrosinated and acetylated tubulin were found to be indistinguishable in MTs in cell bodies and axons, and levels of acetylated α-tubulin were high throughout the neuron, supporting our contention that the original MTs present in the cell body redistributed into the axon during its growth (data not shown). This contrasts with the situation in control neurons, in which the MT polymer at the growing tips of the axons is deficient in acetylated α-tubulin (Baas and Black, 1990). For reasons that are not yet clear, the overall levels of tyrosinated α-tubulin were only marginally diminished compared with controls, and this same result has been obtained in studies on nonneuronal cells whose MTs have been kinetically stabilized by vinblastine (Jordan, M. A., personal communication). One possibility is that the enzymatic process of detyrosination is somewhat less efficient in the presence of the drug.

To address the issue of MT assembly in an entirely different way, we serially reconstructed the MT array of control axons and axons extended in the presence of vinblastine. If MT assembly is active in the axon even in the presence of vinblastine, we would expect that individual MTs would achieve substantial lengths, similar to controls. In contrast, if preassembled MTs translocate into the axon from the cell body, we would expect that the MTs would be significantly shorter than the diameter of the cell body (≈15–20 μm). Our method for serially reconstructing axonal MT arrays is described in Materials and Methods, and serial reconstructions of 20-μm regions of a control axon and an axon grown in the presence of 50 nM vinblastine are shown in Fig. 5. In the control axon, there is only one MT end within the 20-μm region, which is consistent with a MT length exceeding 100 μm (see Bray and Bunge, 1981). By comparison, both ends of every MT appeared within the 20-μm region of the vinblastine axon, permitting us to directly measure their lengths. The average MT length was 1.6 μm, a fraction of that in control axons, and as expected, a fraction of the diameter of the cell body. The average MT length at 16 nM vinblastine, in which we would expect no MT shortening to have occurred, was roughly double that at 50 nM (data not shown), still far

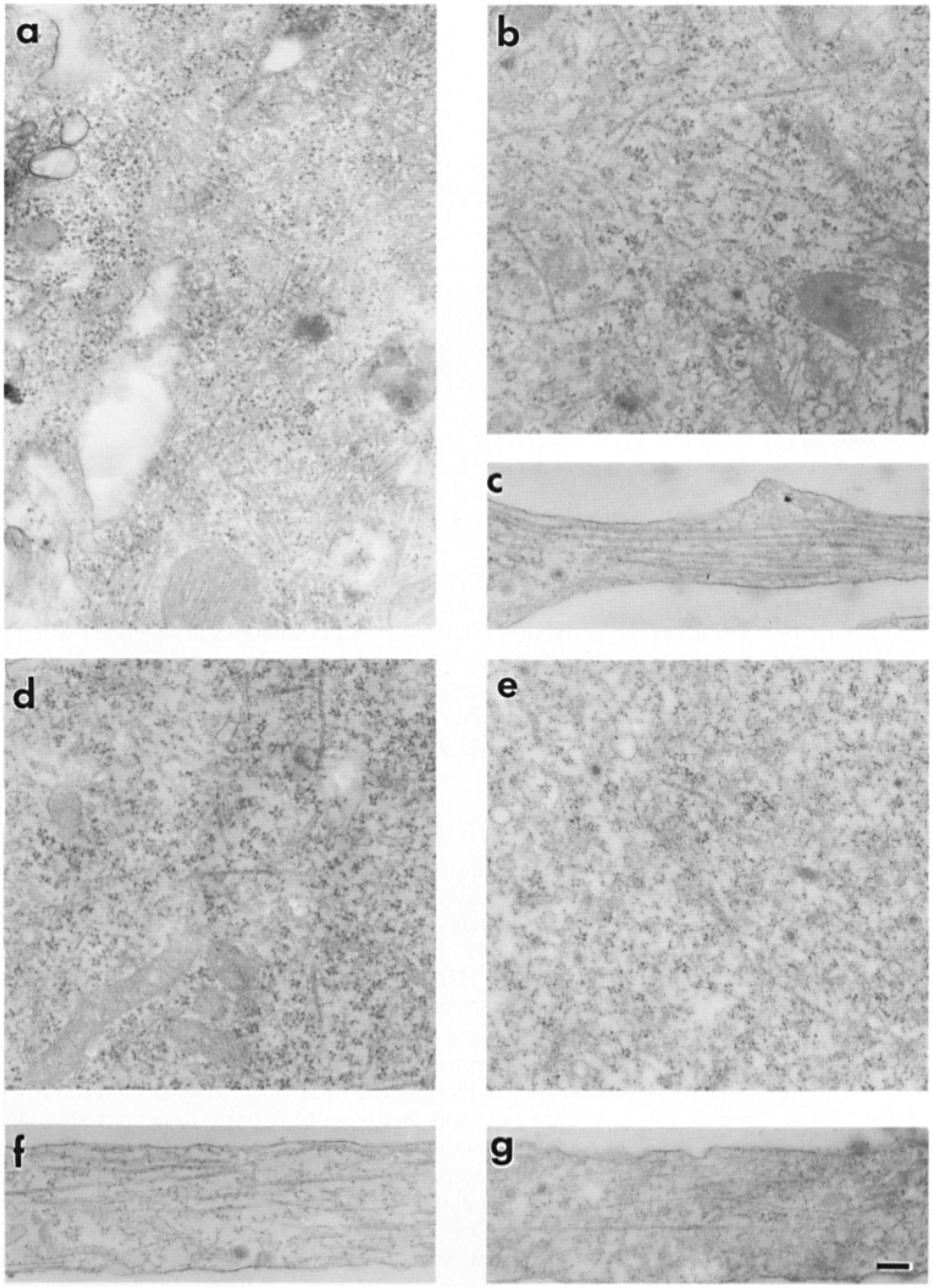

Figure 3. Transmission electron micrographs showing regions of cell bodies and axons. *a* shows a region of the cell body of a freshly plated rat sympathetic neuron before axon outgrowth; *b*, *d*, and *e* show regions of cell bodies of neurons cultured overnight in the presence of 0, 16, and 50 nM vinblastine, respectively; and *c*, *f*, and *g* show regions of axons grown from neurons plated in the presence of these same respective vinblastine concentrations. The cytoplasm of a freshly plated neuron (with no axons) is very dense and contains many MTs. In control overnight cultures, the levels of MTs decrease to less than half, and the axons contain a dense array of MTs. In all of the vinblastine concentrations, the axons contain MTs. With progressively increasing vinblastine concentrations, the overall MT mass in the neuron decreases. There is a progressive increase in MT mass in the axons as they grow, and a corresponding decrease in MT mass from the cell body. These results suggest that there is an active transfer of MTs from the cell body into the axon during its growth, even at the expense of a cell body that cannot replenish itself with new MTs. See Results for more details, and Fig. 4 for quantitative information. Bar, 0.15 μm.

shorter than the length of a MT in a control axon and still a fraction of the diameter of the cell body. Thus all available evidence indicates that concentrations of vinblastine of 16 nM and greater arrest MT assembly (and even promote MT disassembly) in cultured sympathetic neurons, and hence that any MTs present within the axons growing from these neurons are the result of transport from the cell body.

Microtubule Polarity Analyses

Our principal goal was to determine whether the transport properties or assembly properties of axonal MTs account for their uniform polarity orientation. Presently, we have described an experimental system in which the contribution of MT transport to the elaboration of the axonal MT array can be dissected apart from the contribution of MT assembly. We next wished to determine the polarity orientation of MTs in the axons grown under these conditions. If these MTs, like those of control axons, are oriented with plus ends distal to the cell body, we can conclude that the transport properties of axonal MTs account for their distinctive polarity orientation. For MT polarity determination, we used the standard "hooking" protocol. In this method, the neurons are lysed in the presence of a special MT assembly buffer containing exogenous brain tubulin. The exogenous tubulin adds onto existing MTs in the form of lateral sheets that appear as "hooks" on the MTs when viewed in cross section. The curvature of the hook reveals the polarity orientation of the MT; a clockwise hook indicates that the plus end of the MT is directed toward the observer, while a counterclockwise hook indi-

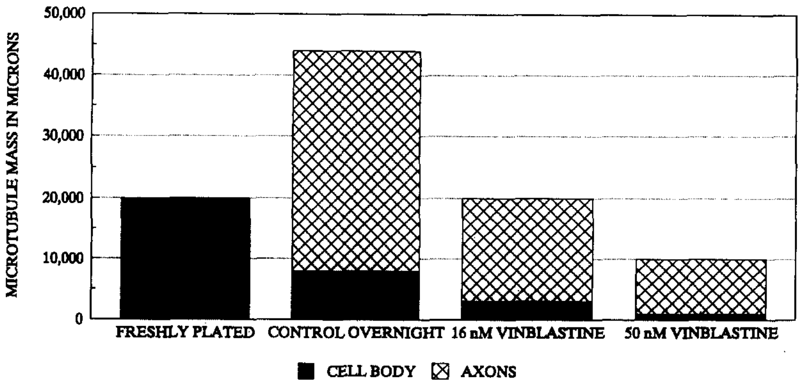

Figure 4. Graph showing the MT mass in freshly plated neurons (with no axons), and neurons plated for 20 h (overnight) in the presence of 0, 16, and 50 nM vinblastine. Methods for quantification are described in Materials and Methods. Black indicates MT mass in the cell body, and hatches indicate MT mass in the axonal arbors. In control cultures, there is a greater than twofold increase in total MT mass relative to freshly plated neurons. This net increase is comprised of a decrease in MT mass from the cell body and a substantial increase in MT mass in the axonal arbor. At 16 nM vinblastine, the total MT mass is indistinguishable from that of freshly plated neurons, indicating that, over this time frame, 16 nM vinblastine acts as a kinetic stabilizer of MTs. Notably, the MT mass of the cell body decreases and that of the axonal arbor is severalfold higher than that of the cell body. At 50 nM vinblastine, the MT distribution is similar to that observed at 16 nM, except that overall levels are less than in freshly plated neurons, indicating that 50 nM vinblastine results in some MT disassembly. Variation in MT masses in axons and cell bodies among the three neurons examined under each condition was exceedingly low, and for this reason, no effort was made to generate error bars. These results indicate that under conditions wherein MT assembly is arrested, and even under conditions that permit some MT assembly, there is an active transfer of MT polymer from the cell body into the axons as they grow. See Results for further details.

cates the opposite. For these studies, we used explant rather than dissociated cultures. Explant cultures are advantageous in that they extend a dense halo of aligned axons, and hence maximize the number of axons that can simultaneously be cross sectioned. Levels of axon outgrowth from explants grown in the different vinblastine concentrations were consistent with levels observed with dissociated cultures (see Fig. 6). In all cases examined – control, 4 nM, 16 nM, 50 nM, and 100 nM – the hooks were predominantly clockwise as viewed from the growth cone, indicating uniform MT polarity orientation, plus ends distal to the cell body (Fig. 6; Table I). Thus even under conditions in which MT assembly has been arrested, and even when there is a net decrease in the MT mass of the neuron, the transport properties of the MTs are sufficient to account for their uniformly plus-end-distal polarity orientation.

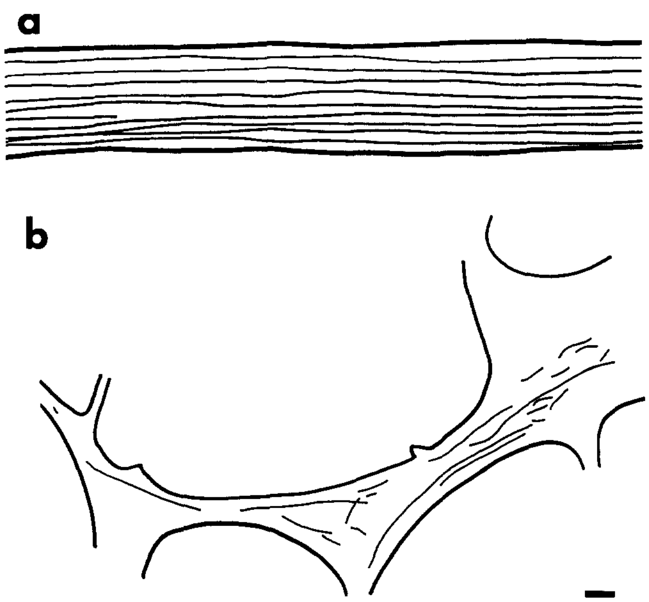

Figure 5. Serial reconstructions of the MT arrays of regions of axons from a control culture in *a*, and a culture grown in the presence of 50 nM vinblastine in *b*. In 20-μm stretches of control axons, the number of MT ends was generally less than three (and in fact, only one in the reconstruction shown here), which is consistent with previous finding of this kind suggesting that axonal MTs generally exceed 100 μm in length (Bray and Bunge, 1981). In axons grown in the presence of 50 nM vinblastine, the MTs generally appear in clusters within the axon, and have an average length of 1.6 μm. At 16 nM vinblastine, the average MT length is roughly double the length at 50 nM (not shown). Bar, 0.8 μm.

Discussion

In the present study, we sought a method which could dissect apart MT transport and assembly, and thereby permit us to investigate their separate contributions to the elaboration of the axonal MT array. We found that we could accomplish this by culturing neurons in the presence of nanomolar levels of vinblastine. At concentrations of the drug which kinetically stabilize MTs in the neuron, and at concentrations which actually favor some MT disassembly, the neurons actively extend axons which contain MTs. In the absence of MT assembly, the only way that these MTs could have arrived in the axon is by movement from the cell body. This is precisely what MT transport means: that a MT that was formerly in one place occupies a different place at a later moment in time. Therefore, by its most fundamental definition, we have demonstrated MT transport from the cell body into the growing axon. This transport is dramatically illustrated not only by the accumulation of MTs in the growing axons, but also by the concomitant depletion of MTs from the cell body. In the presence of the drug, which inhibits the capacity of the cell body to replenish itself with new MTs, the MT mass of the cell body progressively decreases as the MTs move into the axon. These results, which are summarized schematically in Fig. 7, indicate that highly potent and efficient mechanisms exist in the neuron to transport preassembled MTs from the cell body into the axon. These mechanisms are active even at the expense of the cell body, and even under conditions that promote some MT disassembly in the neuron.

The Transport Properties of Axonal Microtubules Establish their Polarity Orientation

We have utilized our newfound ability to analyze MT trans-

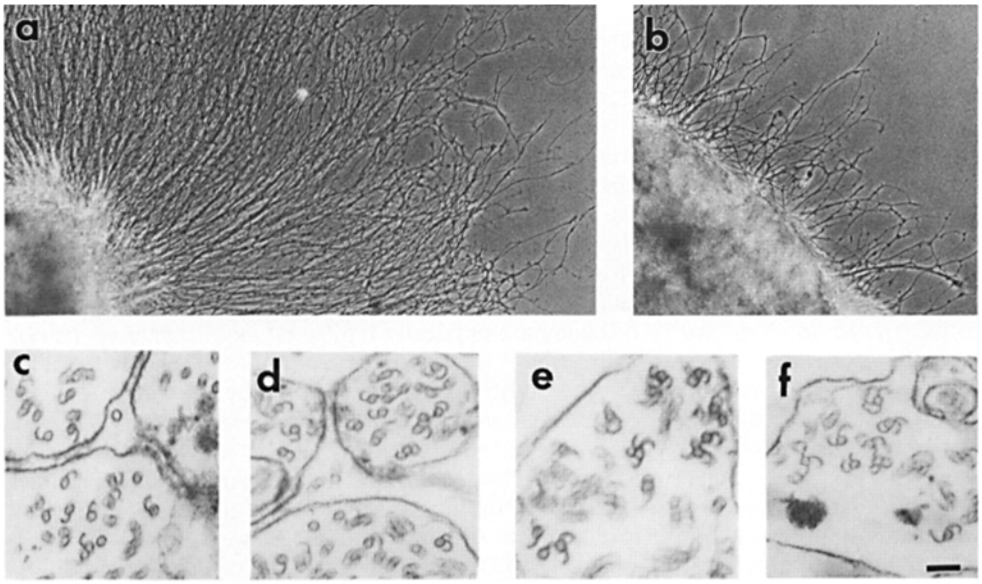

Figure 6. MT polarity analyses on explant cultures grown in the presence of different concentrations of vinblastine. *a* and *b* show explant cultures grown overnight in the presence of 0 and 16 nM vinblastine, respectively. The levels of axonal outgrowth at 16 nM and the other vinblastine concentrations were entirely similar to those observed with the same vinblastine cultures used with the dissociated cultures. *c–f* show MT polarity analyses of axons grown for 20 h in the presence of 0, 16, 50, and 100 nM vinblastine, respectively. Efforts were made to select micrographs showing large numbers of hooks. Therefore these micrographs do not accurately reflect the average levels of MTs in the axons at each drug concentration. In axons grown at all vinblastine concentrations, like control axons, the MT hooks are predominantly clockwise as viewed from the distal tip of the axon, indicating uniform MT polarity orientation, plus ends distal to the cell body. See Table I for quantitative data. Bars: (*a* and *b*) 10 μm; (*c–f*) 0.1 μm.

port properties separately from MT assembly properties to determine whether the transport properties, within themselves, can explain the uniformly plus-end-distal polarity orientation of axonal MTs. Assuming that the polarity orientation of axonal MTs is the direct result of some feature of either their assembly properties or their transport properties (see introduction), then the polarity orientation of MTs in the vinblastine-axons should reveal which of these is correct.

Table I. Microtubule Polarity Orientation in Control and Experimental Axons

	CW*	CCW*	AMB*	UHK*	%HK	%CW
Control	957	43	67	200	84	96
16 nM vinblastine	961	39	50	111	91	96
50 nM vinblastine	980	20	54	125	89	98
100 nM vinblastine	954	46	27	47	96	95

MT polarity orientation was determined using the standard "hook" procedure. In this procedure, neurons are lysed in a special MT assembly buffer in the presence of exogenous brain tubulin. The exogenous tubulin adds onto existing MTs in the form of lateral protofilament sheets that appear on the MTs as hooked appendages when viewed in cross section electron microscopically. A clockwise hook indicates that the plus end of the MT is directed toward the observer, while a counterclockwise hook indicates that the minus end of the MT is directed toward the observer. MT polarity orientation was analyzed in axons elaborated overnight in the presence of 0, 16, 50, and 100 nM vinblastine. Because many of the cross sectional profiles of the axon contained very low numbers of MTs, or were completely devoid of MTs, we scored randomly selected MT profiles until 1,000 unambiguously hooked MTs were tabulated for each condition, rather than keeping track of individual axons. Under all of the conditions examined here, hooks were predominantly clockwise as viewed from the growth cone, indicating uniform MT polarity orientation, plus ends distal to the cell body.

CW, microtubules with clockwise hooks as viewed from tip of the axon looking toward the cell body; *CCW*, microtubules with counterclockwise hooks as viewed from same; *AMB*, microtubules with ambiguous hooks; *UHK*, microtubules with no hooks; *HK*, microtubules with hooks.

* Indicates total of 1,000 MTs scored for each condition.

Our studies clearly indicate that the MTs in the vinblastine-axons are uniformly oriented with their plus ends distal to the cell body, the same orientation as MTs in control axons.

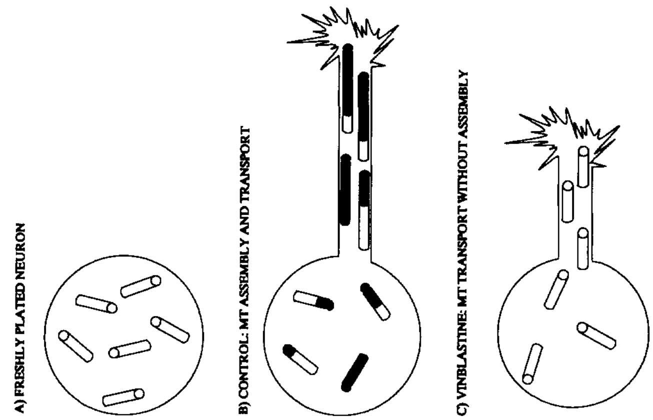

Figure 7. Schematic summary of the results of the present studies. Plus ends of MTs are indicated as open-circled ends, and newly assembled regions of MTs are indicated by blackened regions. A freshly plated neuron contains high levels of MT polymer. Normally, both MT assembly and MT transport are active, and contribute to the growth of the axon. MT assembly lengthens many of the MTs, specifically from their plus ends, as the MTs move down the axon. During this process, the MTs become organized uniformly with plus ends distal to the cell body. No MT assembly occurs in the presence of vinblastine and, as a result, MT transport alone generates the axonal MT array. The MTs remain the same short length as when they were in the cell body (or actually shorten if the vinblastine concentration exceeds 16 nM), and axonal growth is less extensive. However, the MTs are nevertheless uniformly oriented with their plus ends distal to the cell body. These observations indicate that the assembly properties of the MTs are not required to generate the distinctive polarity orientation of axonal MTs. Rather, the transport properties of the MTs, within themselves, establish the uniformly plus-end-distal polarity orientation of axonal MTs.

These results suggest that MTs translocate from the cell body of the neuron into the axon exclusively with their plus ends leading, and that this process is unrelated to the assembly properties of the MTs. Based on these observations, we conclude that the transport properties of the MTs, within themselves, are capable of establishing their distinctive polarity orientation in the axon.

The idea that the transport properties of axonal MTs determine their polarity orientation is consistent with a growing body of information about MT-based transport events within living cells. It is now well recognized that membranous organelles are transported through the cytoplasm along the surface of MTs, and that cytoplasmic motors such as dynein and kinesin provide the directionality for this movement. Dynein moves organelles toward the minus ends of MTs, while kinesin moves organelles toward the plus ends of MTs (for reviews see Brady, 1991; Allan et al., 1991). Given that all motion is relative, it would not be difficult to imagine how similar mechanisms could move MTs through the cytoplasm. In fact, recent studies demonstrate that these molecular motors can cause isolated MTs to move along the surface of glass coverslips (see, e.g., Brady et al., 1982; Vale et al., 1985; Schnapp and Reese, 1989). It is unclear whether motors other than kinesin or dynein exist, but the plus-end-distal polarity orientation of axonal MTs indicates that the motor which moves the MTs has the same directionality as dynein, not kinesin. The greater drag on the MTs, which are significantly longer than the diameter of roundish organelles such as synaptic vesicles, could explain why MTs move through the cytoplasm much more slowly than membranous organelles move along MTs. Substantial efforts will be required to define the specific motor and further characterize the nature of the machinery which transports MTs from the cell body into and down the axon.

What are the specific structures in the cytoplasm against which the MTs move? One possibility is that MTs move relative to a stationary membranous system. This notion is attractive in that it proposes the interplay of virtually the same types of mechanisms that move membranous organelles relative to the MTs. Moreover, studies on the accumulation of organelles after constriction of the axon indicate that the long stretches of ER within the axon do not accumulate at constriction sites, and hence are most probably stationary within the axon (Ellisman and Lindsey, 1983). However, arguing against this possibility is the fact that membranes are fluid and thus may be poorly suited for force generation. Another possibility is that MTs in motion move relative to stationary MTs. In considering this possibility, it is important to note that studies on tubulin transport in the axon have revealed no evidence for the existence of entirely stationary MTs in the axon (Black and Lasek, 1980; Lasek, 1982, 1986, 1988). Thus, if stationary MTs are required for other MTs to move, then it is necessary to hypothesize that any individual MT could be either moving or stationary at any given instant in time. In this view, individual MTs shift between moving and stationary phases because of steric hindrance of their motion, or perhaps because of more specific but nonetheless temporary immobilization due to cross-links with the plasma membrane, internal membranous structures such as ER, or other cytoskeletal structures. Some support for the idea that MTs move relative to other MTs is provided by our results on the vinblastine-axons, in which the MTs never appear to be entirely isolated from other MTs, but rather appear in clusters with one another. Another possibility worth mentioning is that MTs may move relative to neurofilaments, which are transported with very similar kinetics to the MTs themselves (Lasek et al., 1992).

Finally, the characteristics of the axonal MT array generated by transport alone dramatically illustrate the importance of MT assembly in generating a normal axonal MT array. Individual MTs in the vinblastine-axons are severalfold shorter than normal axonal MTs, which can achieve lengths of over 100 μm (Bray and Bunge, 1981). Presumably, the length of the MTs that are transported into the axon from the cell body are physically limited by the diameter of the cell body, even if some curvature of the MTs can occur within the cell body. Other studies from our laboratory strongly suggest that each MT in the axon does, in fact, originate within the cell body, most probably at the centrosome, before its transport into the axon (Baas and Ahmad, 1992; Baas and Joshi, 1992; see introduction). Based on these observations, together with the present findings, we favor the idea that many short MTs originate within the cell body and translocate into the axon, plus-ends-distal. Many of the MTs give up tubulin subunits for the elongation of other MTs, resulting in a shift during transit from large numbers of short MTs to a smaller number of longer MTs (see Lasek, 1986; Ahmad et al., 1993). In this way, MT transport and assembly work together to generate the characteristic MT array of the axon.

In conclusion, there is increasing evidence that both MT transport and assembly are crucial for the elaboration of a normal axonal MT array. We have characterized a novel experimental system for studying the relative contributions of these factors. By culturing neurons under conditions that arrest MT assembly, we can investigate the contribution of MT transport independent from that of assembly. The present efforts using this experimental system indicate that the uniformly plus-end-distal polarity orientation of axonal MTs is established even under conditions wherein a MT array is generated by transport with no assembly. These findings indicate that the transport properties of axonal MTs, within themselves, establish their distinctive polarity orientation. Additional efforts using our experimental system and other experimental systems will be required to further define the unique contributions of MT assembly and transport, and how they interface with one another during the growth of the axon.

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