plies a necessity for subunit loss at the poles, which could play a role in
aphase movement. Some models of spindle dynamics have proposed a continuous
flux of subunits polewards during metaphase, which the the chromatid would then
be during anaphase (5). However, this prediction of a regular flux is
Difficult to reconcile with the observation that individual MTs incorporate
subunits at widely differing rates. This independent behavior of individual MTs
make the flux very difficult to detect by methods which average many MTs,
such as slot photobleaches of spindles labelled with fluorescent tubulin (8).
What then might be the role of an irregular subunit flux? Perhaps it allows the
MTs to remain in a dynamic equilibrium with free tubulin, by continuous
addition and loss of subunits - this could be important for controlling the size
of the spindle, or sensing the correct time to progress into anaphase.

REFERENCES
Microtubules in CHO Cells Following Recovery from a Colcemid Block.
J. Cell Biol. 97: 202-208
Cell Biol. 91: 131s-147s
4) D. Kristofferson, T.J. Mitchison, and M.W. Kirschner (1985) Direct
Observation of Steady State Microtubule Dynamics. J. Cell Biol. (submitted)
6) T.J. Mitchison and M.W. Kirschner (1985) The Kinetochore In Vitro II:
7) E.D. Salmon (1975) Pressure Induced Depolymerisation of Spindle
Microtubules I: Changes in Birefringence and Spindle Length.
Cell Biol. 65: 114-127
using a Fluorescein Labelled Tubulin and measurements of Fluorescence Recovery
9) E.S. Schulze and M.W. Kirschner (1985) Microtubule Dynamics in Interphase

DYNAMIC INSTABILITY AND DIFFERENTIAL STABILITY OF CYTOPLASMIC
MICROTUBULES IN HUMAN MONOCYTES

LYNNE CASSIMERIS, PAT WADEWORTH AND E. D. SALMON
Department of Biology, University of North Carolina, Chapel Hill, N.C.

INTRODUCTION
Our basic experimental approach was to measure the kinetics of microtubule
(Mt) depolymerization at the resolution of individual Mt with the expectation
that this would enable us to evaluate in vivo the probable validity of various
theories of Mt assembly/disassembly (1,2,3,4,5). Addition of a high
concentration of nocodazole (10µg/ml) to the buffer was used to block
elongation of Mt (6,7) that were at steady-state assembly. Nocodazole rapidly
penetrates cells and binds to the colchicine-binding site on the tubulin dimer
(6,8). This site is not exposed when tubulin is polymerized into microtubules
(6,7). In mitotic PkC cultured cells, the great majority of the spindle Mt
(non-kinetochore Mt) depolymerize with a half-time of less than 10 sec
when assembly is abruptly blocked with 10µg/ml nocodazole (9, unpublished
observations). A similar result was previously found when tubulin, complexed
with colchicine, was microinjected into mitotic cells at 2µM or greater
intracellular concentrations (9). In the present experiments, cells were fixed
and permeabilized at various times after nocodazole treatment, and Mt were
visualized by immunofluorescence after labeling with a monoclonal antibody to
tubulin. Using an image-intensified video camera, we traced the profiles of
individual Mt into a computer and the cytoplasmic Mt complex (CMTC) was
reconstructed graphically. Initially, interphase PkC and BSC cells were
examined, but because of the high density of Mt, individual Mt could not be
accurately tracked so only qualitative results were achieved (10). As a
result, our analysis has concentrated on the dynamics of the CMTC in human
monocytes, a cell type for which all Mt can be tracked along their entire
length.

MATERIALS AND METHODS

Monocytes: Human white blood cells were obtained by allowing whole blood to
clot on coverslips that had been precoated with 5% human serum. After 20 min
in a humid chamber at 37°C, the clots were pulled away while immersed in
saline G (2 mM phosphate, 0.137 mM NaCl, 5 mM KCl, 0.6 mM MgSO₄, 0.1 mM CaCl₂, pH 7.3). Coverslips were then rinsed several times in saline G and then returned to a humid chamber at 37°C for 20 min to allow the adherent cells time to spread.

Microtubule Depolymerization and Indirect Immunofluorescence: Coverslips of partially spread monocytes were incubated in a 10 μg/ml solution of nocodazole (Sigma) in saline G at 37°C for various times before fixation. To achieve better resolution of Mts by reducing interference from the tubulin subunit pool (11), cells were lysed for 15 sec in 80 mM PIPES, 5 mM BTPA, 1 mM MgCl₂, pH 6.8, 0.5% Triton X-100. Following lysis, cells were fixed for 10 min in 2% paraformaldehyde, 0.1% glutaraldehyde in PBS, pH 7.3. Coverslips were then rinsed with PBS, extracted in -20°C methanol (6 min) and -20°C acetone (1 min), rehydrated in PBS, rinsed in PBS with 1% BSA, 0.1% Tween 20 and finally rinsed again in PBS. Next, 50 μl of a monoclonal antibody to β-tubulin (12) was then applied to each coverslip, and the coverslips were incubated for 2 hours in a humid chamber at room temperature. After rinsing in PBS, the coverslips were incubated with a 1:25 dilution of rhodamine-conjugated, goat anti-mouse IgG (Cappel Labs) for 2 hours at room temperature. Coverslips were then rinsed in PBS and mounted with Gelvatol (Monsanto).

Microscopy and Length Determinations: Cells were imaged with a 63x planapo lens and a Zeiss Universal microscope equipped for epifluorescence. To determine the length of Mts, we projected the fluorescent image onto a Sanyo T.V. monitor (model VM 420B) through a Venus IV 2 low light level camera and traced the T.V. image onto transparent acetate. It was necessary to focus up and down while tracing in order to follow each Mt completely.

The Mt tracings were then digitized with a HiPad Digitizer (Houston Instrument) and an Apple II Plus computer. We developed a program to calculate histograms of Mt lengths, the mean Mt length (±S.D.), total Mt length (total polymer ±S.D.) and the number of Mts per cell. The image of the digitized cell was also drawn on the computer screen and printed, along with the above data, with an Epson model MX-80F/T printer. At each time point, 13 to 19 cells were analysed; the total number of Mts measured was 1,258.

Figure 1: Fluorescence (a) and phase contrast (b) micrographs of a human monocyte after immunofluorescent staining with a monoclonal antibody to tubulin. Bar= 2 μm.

RESULTS

Figure 1 shows the typical Mt distribution in a control monocyte; a digitized image of the Mt distribution in another control monocyte is illustrated in Figure 2. All Mts appeared to extend radially away from the centrosome. Many end near the cell periphery, but a few extend along the cell perimeter. The average number of Mts per cell was 271 ± 41 (n = 29 cells). The average length of a Mt in a cell was 6.5 ± 1.1 μm (n = 786 Mts) and the total polymer/cell was 176 ± 38 μm. Mt lengths may be underestimated by about 5% because of projection errors due to the 2 μm cell height.

There were four main features in the response of the CMTK when Mt elongation was abruptly blocked by nocodazole, as can be seen in Figures 2, 3, and 4. First, the initial phase of Mt depolymerization was rapid; the half-time of total polymer loss and microtubule number per cell was about 40 sec. Second, the average length of persistent Mts remained relatively constant during the depolymerization process. Third, the distribution of Mt lengths remained relatively constant and fourth, a minor fraction of the initial Mt population was differentially stable compared to the majority of labile Mts. The effects of nocodazole were reversible (8). Within 20 min following washout of nocodazole, Mt number and total polymer returned to about 80% of the initial values (data not shown).

Initial studies using cold temperature (3°C) to induce disassembly showed a similar, but slower, pattern of Mt loss. Again, there was a
concurrent decrease in total polymer and number of Mts with a much slower decrease in the average length of persisting Mts (data not shown).

**Analysis and Discussion**

Our conclusion from these studies is that the kinetics of disassembly of the CMTC in monocytes is governed by asynchronous initiation of catastrophic depolymerization among two subpopulations of Mts, the labile and differentially stable classes. As shown in the histograms in Figure 3, no short intermediates of depolymerizing Mts were seen. To determine if short Mt fragments were lost during the lysis step before fixation, cells were treated for 30 sec in nocodazole and fixed for immunofluorescence without prior lysis. Although there was considerable background staining due to the unextracted tubulin pool, no short Mts were observed. Thus, we conclude that once initiated, depolymerization of an individual Mt is extremely rapid and complete. The dashed curves in Fig. 4 represent the theoretical shift in the length distribution of Mts if all the Mts shortened simultaneously at the same constant rate. The rate was chosen to match the observed 50% reduction in Mt polymer within 40 sec. This model predicts a shift of the length distribution towards the origin, which is clearly not shown by the data. Preliminary analysis of the kinetic curves in Figure 4 show that 60% of the initial Mt population is labile and disappears 20 times faster than a differentially stable 40% of the initial Mt population (data not shown). However, even with two populations of Mts, which shorten at a 20 fold difference in rate, short Mt intermediates are predicted for simultaneous depolymerization. Such short Mts are not seen in the length histogram data. Because no intermediates of Mt depolymerization have been seen, our data provides no evidence on the sites of tubulin dissociation within a Mt, once depolymerization is initiated.

**Figure 3:** The changes in total polymer (a), number of Mts (b), and average Mt length per cell (c) following incubation in nocodazole. Error bars at each data point correspond to the standard deviation of the mean.

**Figure 4:** Histograms of average Mt length distributions per cell which were lysed and fixed following incubation in nocodazole at the times given. The arrows denote the mean of each distribution. The dashed lines are the result of a theoretical model as described in the text.
We can only estimate a lower limit for the tubulin dissociation constant for depolymerizing labile Mts. In 40 sec, 50% of the Mts have disappeared. A tubulin dissociation rate of 264 dimers/sec/Mt is required for depolymerization of a 6.5μm long Mt in 40 sec. Since initiation of Mt depolymerization occurs continuously over this 40 sec interval and no short intermediate lengths were seen, the rate of tubulin dissociation is likely to be much higher. This rate is similar to the value determined for the labile, non-kinetochoore spindle Mts (9). This similarity indicates that the rate of initiation of depolymerization is likely to be the major difference in the dynamic properties of these different Mt arrays.

The asynchrony in the initiation of the catastrophic Mt shortening shown here can explain the rapid, uniform rate of decay of birefringence which occurs throughout the spindle following injection of colchicine (9) and the rapid, uniform rate of incorporation of fluorescently labeled tubulin into spindle fibers at steady-state as shown by fluorescence redistribution after laser photobleaching (FRAP) in both sea urchin embryos and mammalian cultured cells (13,14). This pattern of Mt behavior at steady-state assembly also explains the slower, but uniform recovery of fluorescence after laser photobleaching of fluorescently labeled interphase Mt arrays in living cells(14).

The dynamic behavior of the Mts in the monocyte CMTC reported here provides substantial support for the Dynamic Instability model (3,4). In this model, Mts at steady-state assembly are proposed to exist in persistent phases of either nucleated growth or catastrophic, rapid shortening, with infrequent transitions between phases. Mt instability or phase transitions are proposed to depend on hydrolysis of GTP ligated to tubulin. At variable times following tubulin incorporation onto a growing end of a Mt, ligated GTP is hydrolyzed to GDP forming a Mt core of tubulin-GDP capped at the growing end(s) by tubulin-GTP. When GTP hydrolysis catches up with growth, the tubulin-GDP core is exposed, and rapid, catastrophic depolymerization ensues. Among a population of Mts, loss of the tubulin-GTP caps could be asynchronous, leading to the asynchronous initiation of catastrophic Mt depolymerization reported here for the monocytes. However, the origins of the dynamic instability behavior of Mts in vivo is likely to be more complex than the simple tubulin-GTP cap model predicts. For example, Mts become stable when cytoplasmic ATP concentrations are reduced by metabolic inhibitors (8,15), a condition which is expected to decrease GTP concentration and expose the tubulin-GDP core. The Dynamic Instability model (3,4) also does not predict the two classes of differentially stable Mts observed in the monocytes and in the CMTC of other cultured cells (8,11,16,17). Although differences in tubulin species may account in part for Mt heterogeneity (16,17), the cooperative, phosphorylation-dependent (18) binding of microtubule associated proteins to the walls or ends of Mts probably plays a key role in the origins of the dynamic instability and differential stability among a population of Mts.

ACKNOWLEDGMENTS

We thank Cate Parker for her photographic skills, Winthrop Jackman for his beautiful monocytes, David Newton for programming, Nancy Pryer for her suggestions and criticisms, Nancy Salmon for her editorial help and Marc DeBrabander for his infinite patience. The monoclonal antibody to tubulin was provided by B. Neighbors in Dick McIntosh's lab. Supported by NIH 24364 and NIH RR07072 to EDS.

REFERENCES