Passive Mechanical Behavior of Human Neutrophils: Effects of Colchicine and Paclitaxel

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ABSTRACT The role of microtubules in determining the mechanical rigidity of neutrophils was assessed. Neutrophils were treated with colchicine to disrupt microtubules, or with paclitaxel to promote formation of microtubules. Paclitaxel caused an increase in the number of microtubules in the cells as assessed by immunofluorescence, but it had no effect on the presence or organization of actin filaments or on cellular mechanical properties. Colchicine at concentrations <1.0 μM caused disruption of microtubular structures, but had little effect on either F-actin or on cellular mechanical properties. Higher concentrations of colchicine disrupted microtubular structure, but also caused increased actin polymerization and increases in cell rigidity. Treatment with 10 μM colchicine increased F-actin content by 17%, the characteristic cellular viscosity by 30%, the dependence of viscosity on shear rate by 10%, and the cortical tension by 18%. At 100 μM colchicine the corresponding increases were F-actin, 25%; characteristic viscosity, 50%; dependence of viscosity on shear rate, 20%; and cortical tension, 21%. These results indicate that microtubules have little influence on the mechanical properties of neutrophils, and that increases in cellular rigidity caused by high concentrations of colchicine are due to a secondary effect that triggers actin polymerization. This study supports the conclusion that actin filaments are the primary structural determinants of neutrophil mechanical properties.

INTRODUCTION

Leukocytes play a vital role in host defense against invading microorganisms and parasites. They circulate in the blood until they receive specific signals that activate their biological machinery. Neutrophils can also have a significant influence on blood flow and oxygen delivery in the microvasculature because of their large volume and low deformability. It has been shown that an increase in cell concentration or a decrease in cellular deformability significantly elevates flow resistance in the capillary networks (Braide et al., 1984; Harris and Skalak, 1993a, b). The low deformability of neutrophils has been indicated as a major mechanism for neutrophil sequestering in the lungs (Wiggs et al., 1994). Leukocyte deformability has been implicated in the pathophysiology of a number of diseases. For example, leukocytes have been found to block the capillaries after hemorrhagic shock or ischemia, preventing full restoration of blood flow in the microvasculature and resulting in concomitant tissue damage (Bagge et al., 1980; Engler et al., 1983). Leukocyte deformability has been reported to decrease after infarction or stroke, affecting nutritive flow in exchange vessels and contributing to local ischemia and tissue necrosis after stroke (Ernst et al., 1987; Mercuri et al., 1989). Rigidification of the cells has also been reported in diabetes, possibly contributing to microcirculatory complications in diabetic patients (Ernst and Matrai, 1986; Vermes et al., 1987). In some forms of leukemia leukocyte concentration in the peripheral blood is elevated and a significant portion is immature cells, which are less deformable than normal mature cells (Lichtman and Kearney, 1976; Tsai et al., 1996a). Thus, these cells have a high propensity to impair blood flow and oxygen delivery, resulting in severe circulatory complications (Lichtman and Rowe, 1982; Rowe and Lichtman, 1984).

Although the importance of leukocyte mechanical properties in the pathophysiology of various diseases has been increasingly recognized, the regulation of leukocyte mechanical properties is still not well understood. In former studies it has been shown that actin filaments play a major role in regulating the mechanical properties of passive neutrophils (Tsai et al., 1994). Microtubules have also been shown to make significant contributions to the mechanical properties of cells. In particular, disruption of microtubules has been shown to dramatically lower the cytoplasmic viscosity of proliferating immature leukocytes, based on the model of the human promyelocytic leukemia cell line HL-60 cells (Tsai et al., 1996a). However, when HL-60 cells are induced to differentiate to mature cells, the ultrastructure of the cytoskeleton changes (Meyer and Howard, 1983, 1987; Erzurum et al., 1991; Hallows and Frank, 1992) and their mechanical properties change as well (Hallows and Frank, 1992; Tsai et al., 1996b). It is possible that the relative contributions from the microtubules may change with cellular differentiation and maturation. However, even in mature neutrophils, it has been reported that depolymerization of microtubules can significantly reduce the mechanical stiffness of leukocytes (Chien and Sung, 1984).

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In contrast, immunofluorescence microscopic studies revealed that microtubules in mature leukocytes (neutrophils and monocytes) are relatively few in number in comparison with the microfilaments (F-actin) (Anderson et al., 1982; Cassimeris et al., 1986). The microtubules are mainly restricted to the cell body, even though they do penetrate the actin-rich peripheral cytoplasm to some extent. Chemicals that affect tubulin assembly, such as colchicine, vinblastine, podophyllotoxin, and vinca alkaloids, have been used to assess the importance of microtubules in leukocyte function. These agents inhibit tubulin polymerization and cause a net decrease in the number of cytoskeletal microtubules. However, treatment of cells with these reagents does not have profound inhibitory effects on the mechanical function of leukocytes, i.e., chemotaxis and phagocytosis, in sharp contrast to the F-actin severing toxins, the cytochalasins (Keller et al., 1984).

In this study we investigated the specific role that microtubules may play in determining the mechanical properties of human neutrophils when they are subjected to large deformations similar to those that the cells undergo in the microvasculature. The assembly of microtubules was altered using the reagent colchicine to inhibit tubulin polymerization or paclitaxel to stabilize microtubules and enhance tubulin polymerization. Conformational changes in the cytoskeleton of the cells induced by the reagents were examined by fluorescence microscopy and quantified by flow cytometry. Changes in the mechanical properties of the cells were quantitatively assessed by single-cell micropipette aspiration. Our results indicate that ultrastructural changes in the microtubule network alone do not significantly affect the mechanical properties of passive neutrophils, but disruption of microtubules by colchicine may have a secondary effect on the cytoskeleton that triggers actin polymerization and cell activation, resulting in rigidification of the cells. These results further support the hypothesis that microfilaments (F-actin) play the predominate role in determining the mechanical properties of passive neutrophils.

MATERIALS AND METHODS

Neutrophil preparation

The isolation of human neutrophils has been described in detail elsewhere (Tsai et al., 1993). Peripheral blood was donated by normal healthy adults after informed consent. Two tubes of 10-ml whole blood were drawn in sterile tubes containing sodium heparin as an anti-coagulant (Vacutainer, Becton Dickinson, Rutherford, NJ). Each 3.5 ml of whole blood was layered on top of 3.0 ml of Ficoll-Hypaque density gradient (NIM, Cardinal Associates, Santa Fe, NM) in a centrifuge tube and spun at 800 $\times$ g for 30–40 min. The polymorphonuclear leukocyte pellet (>95% neutrophils) was collected with a long spinal needle and suspended in phosphate buffered saline without Ca^{2+} or Mg^{2+} (PBS) (BioWhittaker, Walkersville, MD). Cells were washed twice with fresh PBS after separation and resuspended in PBS with 5% fetal bovine serum (FBS) (HyClone Laboratory, Logan, UT). Micromechanical experiments were usually completed within 7 to 8 h after the blood was drawn.

Alteration of the cytoskeletal microtubules

To alter the cytoskeletal microtubules, neutrophils were treated with 0.5–2.5 μM paclitaxel (Taxol) to promote polymerization of tubulin into microtubules or 0.1 to 100 μM colchicine to collapse cellular microtubules. Colchicine and paclitaxel were purchased from Sigma Chemical (St. Louis, MO). One mg paclitaxel was dissolved in 1.171 ml DMSO to produce a 1 mM stock solution. The stock was kept frozen at −20°C before the experiment. A stock solution of 20.2 mM colchicine was made by dissolving 25 mg colchicine in 3.098 ml deionized water and kept in the refrigerator at 4°C. When the reagents were used for an experiment, the stock solutions were warmed to room temperature and diluted in fresh PBS buffer to twice the desired concentrations. An equal dilution (v/v) with the cell suspension yielded the desired final drug concentrations. Micromechanical measurements were carried out after the cells had been exposed to the drugs for at least 20 min and were completed within 90 min.

Microscopy of cell morphology

Morphological changes induced by the reagents colchicine and paclitaxel were examined on an inverted microscope (Diaphot, Nikon, Melville, NY) under bright field (Koeller) illumination. After the drug treatments a cell suspension was placed in a U-shaped microchamber and mounted on the microscope stage. The morphological changes of the cells were examined using a Nikon 60× objective. Bright field images of the cells were photographed onto Kodak T-Max film (ISO 400, Eastman Kodak, Rochester, NY) with a Nikon FM-2 35-mm camera and archived in Kodak PhotoCD format for analysis.

Micromechanical measurements

Cell cortical tension was assessed by measuring the minimal pressure, Δp_{cr}, to deform a cell into a hemisphere inside a micropipette (Tsai et al., 1994). To determine Δp_{cr} a small pressure was applied and maintained for 2 min for the cell to fully respond. The pressure was then increased 2.45 Pa (0.25 mm H_2O) and maintained for 2 min for the cell to deform. This procedure was repeated until the cell was deformed into a hemisphere and the projection inside the pipette remained stationary. The pressure at this point was measured as the threshold pressure Δp_{cr}. The pressure was further increased by additional one or two steps of 2.45 Pa to ensure that the cell did not adhere to the pipette wall. According to Laplace's law,

$$T = \Delta p_{cr} \cdot R_p R_c / 2(R_c - R_p), \quad (1)$$

where T is the cell cortical tension and R_p and R_c are pipette and cell radii, respectively.

Cellular viscosity was measured with single-cell pipette aspiration as described previously (Tsai et al., 1993). Individual spherical cells were selected and drawn into a 4.2-μm pipette at aspiration pressures ranging from 0.3 to 1.6 kPa. The process of cell entry into the micropipette was monitored on a television monitor and video tape-recorded. Cell entry was analyzed as a function of time and aspiration pressure on a computer-based imaging processing system (DataCube, Peabody, MA). The apparent viscosity of neutrophils and the mean shear rate during the course of cell entry were calculated according to a numerical analysis of a single cell entering a micropipette (Tsai et al., 1993). The non-Newtonian behavior of the cells was further characterized based on the power-law fluid model (Tsai et al., 1993).

Fluorescence microscopy of cytoskeletal microtubules

Protocols for observing changes in microtubule organization using immunofluorescence microscopy were developed based on published procedures (Anderson et al., 1982; Safiejko-Mroczka and Bell, 1996). After treatments with the drugs for ~20 min, neutrophils were fixed in 3.2% paraformal-

dehyde (PFA) in PBS and stored at 4°C overnight. Fixed cells were washed with PBS and cross-linked with 1 mM dithiobis (succinimidyl propionate) (DSP) (Pierce, Rockford, IL) in microtubule stabilizing buffer (MTSB) (1 mM EGTA, 4% polyethylene glycol 8000, 100 mM PIPES, pH 6.9) at 37°C for 10 min. Cells were then extracted with 0.5% Triton X-100 in MTSB (TSB) in the presence of 1 mM DSP at 37°C for 10 min, and further extracted with TBS without DSP at 37°C for 10 min. The extracted cells were rinsed with PBS for 5 min and incubated with 0.1 M glycine in PBS for 5 min at room temperature. Cells were washed with 1% bovine serum albumin in PBS (PBS-BSA), and stained with Cy3-conjugated monoclonal anti-β-tubulin antibody (Clone TUB 2.1) (Sigma) in the dark for 45–60 min at room temperature. Stained cells were washed with PBS-BSA and suspended at 5×10^5 cells/ml for microtubule analysis.

Ultrastructural changes in microtubules were examined by epi-fluorescence microscopy on a Nikon Diaphot inverted microscope with a Nikon 100× oil-immersion objective (NA, 1.4). The fluorescent images of the cells were photographed on Kodak Ektachrome films (ISO 1600) and archived in PhotoCD format for analysis. Content of intracellular microtubules was quantified based on the fluorescence intensity. Fluorescent images of single cells were amplified with a video intensifier (GENSYS II, Dage-MTI, Michigan City, IN) and an integrating CCD camera (CCD-72/DSP 2000, Dage-MTI). The integrated images were digitized via a video frame grabber board (Vision-EZ, Data Translation, Marlboro, MA) and stored on a computer hard disk for analysis. For each sample 20–25 cells were measured.

Flow cytometry and single-cell assay of intracellular F-actin

After the drug treatments, neutrophils were fixed and stored in 3.2% PFA at 4°C overnight for F-actin analysis by fluorescence microscopy and flow cytometry. The changes in neutrophil intracellular F-actin were measured by the method of Wallace et al. (1987). The intracellular F-actin was labeled with the fluorescent probe BODIPY FL-phallacidin (Molecular Probes, Eugene, OR), which specifically binds to F-actin filaments, but not monomeric G-actin, in a stoichiometric ratio of one phallacidin per actin subunit (De La Cruz and Pollard, 1994). Fixed cells were washed twice with PBS-BSA and incubated in PBS containing 165 nM BODIPY FL-conjugated phallacidin in the dark for 45–60 min at room temperature. Labeled cells were rinsed with PBS-BSA and suspended at 5×10^5 cells/ml for cytoskeletal analysis.

The quantification of neutrophil intracellular F-actin content was carried out on a Coulter Elite ESP flow cytometer (Coulter Electronics, Miami, FL). The fluorophore BODIPY FL was excited at a wavelength of 488 nm with a 25-mW argon laser and the emitted fluorescence by BODIPY FL was collected at a wavelength of 520–530 nm. A small population of red blood cells in each sample was excluded from the measured population based on their low level of forward light scattering and their low content of F-actin. The intracellular F-actin was assumed to be directly proportional to the fluorescence intensity. Changes induced by the reagents were expressed as a percentage relative to matched control cells.

Changes in the spatial distribution of intracellular F-actin were examined by fluorescence microscopy. The fluorescence was excited at a wavelength of 490 nm and viewed with a Nikon 100× oil-immersion objective. To better correlate the fluorescence measurements with the micromechanical measurements, 20 spherical cells from each sample were specifically selected under red light (bright field), and fluorescence images of these cells were captured as described. The total fluorescence of each cell was calculated from the gray scale of the digitized images.

RESULTS

Effect of colchicine and paclitaxel on neutrophil morphology

The striking effects of the microtubule reagents were first evident from the morphological changes of the cells observed with bright field microscopy. Shown in Fig. 1 are typical micrographs of the morphology exhibited by normal, paclitaxel-treated, and colchicine-treated cells. The reagent paclitaxel, at a concentration up to 2.5 μM, did not induce any significant changes in cell morphology. Paclitaxel-treated cells (Fig. 1 *b*) maintained the same spherical geometry as normal (untreated) resting neutrophils (Fig. 1 *a*). In contrast, the reagent colchicine activated and polarized neutrophils in a dose-dependent fashion (Fig. 1, *c–f*). Cells treated with 0.1 μM colchicine did not show any visible difference from the control cells (Fig. 1 *c*). The effect of 1 μM colchicine was also very limited, even though a very small number of cells exhibited some minor irregularity in morphology (Fig. 1 *d*). After treatment with 10 or 100 μM colchicine, the majority of the cells were activated and formed pseudopodia (Fig. 1, *e* and *f*). Despite the severe activation by 10 or 100 μM colchicine, a small population of spherical cells remained (*arrows*, Fig. 1, *e* and *f*), which were suitable for rheological measurements.

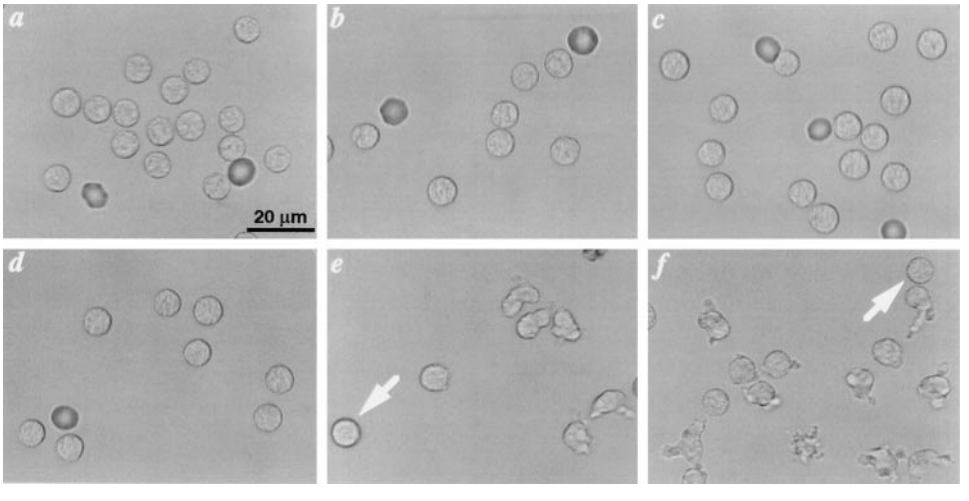

FIGURE 1 Micrographs of the morphology exhibited by normal, paclitaxel-treated, and colchicine-treated cells. (*a*) Normal neutrophils; (*b*) cells treated with 2.5 μM paclitaxel; (*c–f*) cells treated with 0.1, 1, 10, and 100 μM colchicine, respectively. The arrows point to spherical cells that would be suitable for mechanical measurement. Treatment of paclitaxel, up to 2.5 μM, had no apparent effect on cell morphology, whereas colchicine activated and polarized the cells in concentrations of 10 μM or higher.

Effects of colchicine and paclitaxel on neutrophil cortical tension

Alterations of microtubule ultrastructure with paclitaxel or low concentrations of colchicine had no effect on the neutrophil cortical tension. Between 20 and 25 cells were measured at each set of conditions. The mean values of the cortical tension (T) for untreated cells, cells treated with 0.5 μM or 2.5 μM paclitaxel, and cells treated with 0.1 μM or 1 μM colchicine were all statistically indistinguishable (Student's t-test), with mean values of $T \approx 0.0021 \pm 0.0003$ mN/m. At higher concentrations of colchicine, the mean value of the cortical tension increased by ~20%: at 10 μM colchicine, $T = 0.0025 \pm 0.0003$ mN/m; at 100 μM colchicine, $T = 0.0026 \pm 0.003$ mN/m. These increases were statistically significant ($p < 0.01$).

Effects of colchicine and paclitaxel on neutrophil rheology

The effects of the reagents colchicine and paclitaxel on the rheological properties of neutrophils were assessed by single-cell micropipette aspiration. Shown in Fig. 2 are the effects of the two reagents on the apparent viscosity of neutrophils as a function of aspiration pressure. Stabilization of microtubules by the reagent paclitaxel did not result in any significant changes in the cellular properties of neutrophils (Fig. 2 *a*). In contrast, treatment with colchicine increased the resistance of the cells to flow into the micropipette in a dose-dependent fashion (Fig. 2 *b*). To account for possible differences in cell size, the cellular resistance to flow is expressed in terms of an apparent cellular viscosity (μ) calculated from the measured cell entry time and cell dimensions (Tsai et al., 1993). Ten and 100 μM colchicine increased the apparent viscosity by 30–60% and 45–110%, respectively, compared at the same aspiration pressures.

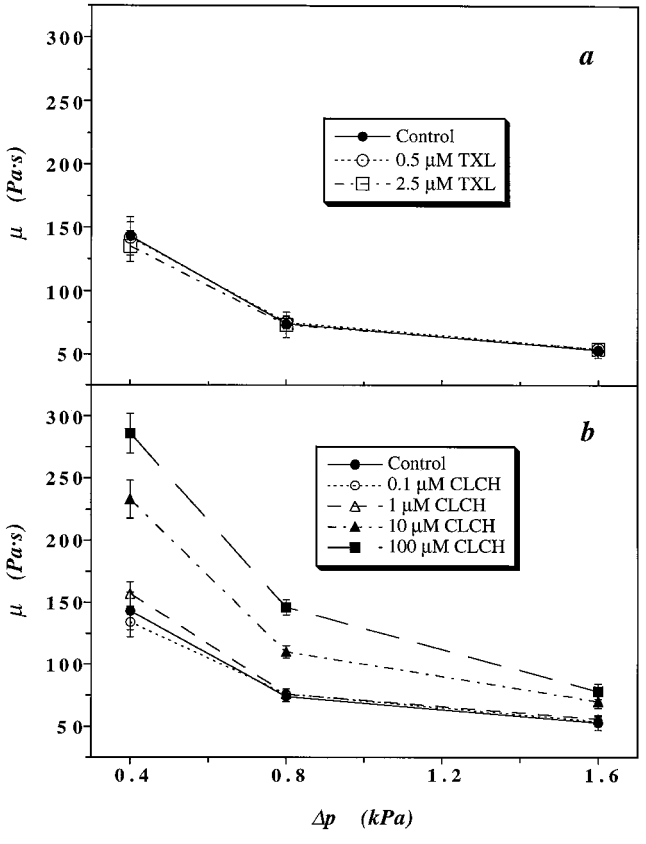

FIGURE 2 Effects of paclitaxel and colchicine on the cytoplasmic viscosity of neutrophils at different aspiration pressures. Paclitaxel, at a concentration up to 2.5 μM, did not affect the cellular deformability of human neutrophils, whereas colchicine rigidified the cells in a dose-dependent fashion.

Consistent with our previous reports (Tsai et al., 1993, 1996a), the apparent viscosity of the cells exhibited a strong dependence on the rate of cellular deformation during cell entry into the micropipette (Fig. 2). Comparison of the data at the same shear rate provides a more rigorous measure of the effects of the microtubule reagents. The non-Newtonian behavior of the neutrophil cytosol is characterized as that of a power-law fluid:

$$\mu = \mu_c(\dot{\gamma}_m/\dot{\gamma}_c)^{-b} \quad (2)$$

where μ is the apparent cellular viscosity, $\dot{\gamma}_m$ is the mean shear rate during cell entry into the micropipette, μ_c is the characteristic viscosity at the characteristic shear rate $\dot{\gamma}_c$, and b is a material constant (Tsai et al., 1993). As in experiments in which the cortical tension was measured, treatment of cells with paclitaxel or with low concentrations of colchicine had no statistically significant effect on the cell mechanical properties. The mean value for the characteristic viscosity μ_c of control samples ($\dot{\gamma}_c = 1\ s^{-1}$) was 160 ± 6 Pa · s, and mean values for μ_c of treated samples (cells treated with 0.5 μM or 2.5 μM paclitaxel and cells treated with 0.1 μM or 1 μM colchicine) ranged from 154 to 170 Pa · s. The mean value for the shear rate dependence coefficient b of control samples was 0.43 ± 0.02, and mean values for b of treated samples (cells treated with 0.5 μM or 2.5 μM paclitaxel and cells treated with 0.1 μM or 1 μM colchicine) ranged from 0.40 to 0.43. However, higher concentrations of colchicine increased both the characteristic viscosity coefficient μ_c and the dependence of viscosity on shear rate, as reflected in the material constant b. Treatment with 10 μM or 100 μM colchicine raised the characteristic viscosity by 30% and 50%, and increased the shear rate-dependence by 10% and 20%, respectively.

Effects of colchicine and paclitaxel on microtubules

The effects of the reagents on neutrophil microtubules were examined by epi-fluorescence microscopy. The ultrastructure of microtubules was visualized using a Cy3-conjugated anti-β-tubulin monoclonal antibody (clone TUB 2.1) (Gozes and Barnstable, 1982). Shown in Fig. 3 are typical fluorescent micrographs of microtubule ultrastructure in normal cells and cells treated with DMSO, paclitaxel, or

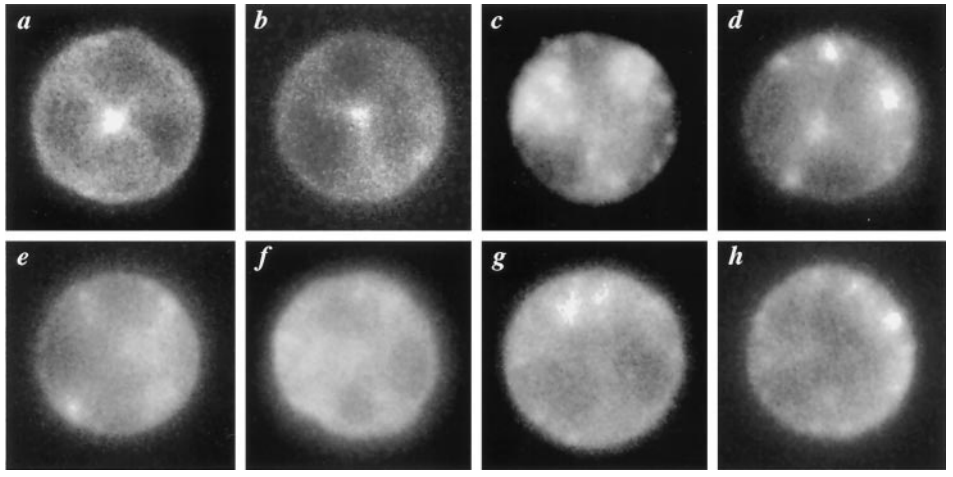

FIGURE 3 Effects of paclitaxel and colchicine on the ultrastructure of microtubules in permeabilized neutrophils observed with epi-fluorescence microscopy. (*a*) Normal neutrophil; (*b*) cell treated with 0.05% DMSO; (*c* and *d*) cell treated with 0.5 and 2.5 μM paclitaxel, respectively; (*e–h*) cells treated with 0.1, 1, 10, and 100 μM colchicine, respectively. Paclitaxel caused an increase in microtubule-associated fluorescence compared to untreated or DMSO-treated cells. Colchicine caused the disappearance of the normal distribution of tubulin fluorescence. Although it may be difficult to tell from these panels, quantitative gray scale measurements of fluorescent images revealed a reduction in fluorescence intensity in colchicine-treated cells (see Table 1).

colchicine. In normal cells, microtubules were visible as spokelike structures, radiating from locations near the cell center to the periphery of the cells (Fig. 3 *a*). A similar organization of microtubules was observed in cells treated with 0.25% DMSO, indicating that the solvent of paclitaxel had no significant effect on microtubules (Fig. 3 *b*). Microtubule reagents paclitaxel and colchicine, however, resulted in dramatic changes in microtubule ultrastructure. When neutrophils were treated with 0.5–2.5 μM paclitaxel, a significant amount of tubulin was polymerized into microtubules and clustered into microtubule bundles, visible as fluorescence-intense hot spots (Fig. 3, *c* and *d*). However, the original organization of microtubules seemed to be well preserved, and individual filaments were still seen to branch from the center to the periphery of the cells. In contrast, colchicine affected the organization of microtubules at all concentrations tested. Generally, the radiating pattern observed in control cells was changed to a more diffuse distribution, and punctate fluorescence at the cell periphery was observed, particularly at higher concentrations (Fig. 3, *e–h*).

The effects of the reagents on neutrophil microtubules were further quantified based on the fluorescence intensity of the cells. To correlate with our mechanical measurements, spherical cells were selected and measured on a single-cell basis. In our protocol of microtubule fluorescence microscopy, neutrophils were permeabilized with 0.5% Triton X-100 detergent. The permeabilization would permit intracellular tubulin monomers to diffuse out of the cells, leaving only polymerized tubulin in the cells. Thus, the fluorescence intensity measured should reflect relative changes in polymerization state of intracellular microtubules. As summarized in Table 1, paclitaxel-treated neutrophils exhibited brighter fluorescence of tubulin than control cells ($p < 0.005$), whereas no significant changes were observed in DMSO-treated cells ($p > 0.4$). These results indicate that the reagent paclitaxel specifically polymerizes tubulin and increases the intracellular pool of microtubules at the concentrations tested. In contrast, the fluorescence in colchicine-treated cells was significantly dimmer than in normal cells and the intensity was reduced from 11% to 35% in cells treated with colchicine at various concentrations from 0.1 μM to 10 μM ($p < 0.005$) (Table 1). However, the fluorescence intensity reduction was only ~6% in cells treated with 100 μM colchicine. This may have been due to trapping of tubulin because of increased actin polymerization at the cell periphery. Most of the fluorescence intensity in these cells was located in "hot spots" near the cell edge, a pattern clearly distinct from that found in untreated cells (Fig. 3).

Effects of colchicine and paclitaxel on F-actin

To provide further insight as to the mechanisms by which these reagents affect neutrophil rheological properties, we

TABLE 1 Effects of paclitaxel and colchicine on fluorescence intensity of intracellular tubulin in permeabilized neutrophils measured by single-cell fluorescence microscopy

Drug Treatment	Fluorescence	Δ (%)	Student's *t*-test
Control	96.7 ± 11.9		
0.25% DMSO	92.8 ± 17.3	N.S.	$p > 0.4$
0.5 μM Paclitaxel	116.3 ± 15.7	20.3 ± 16.2	$p < 0.005$
2.5 μM Paclitaxel	114.2 ± 12.5	18.1 ± 12.9	$p < 0.005$
0.1 μM Colchicine	85.9 ± 8.1	−11.2 ± 8.4	$p < 0.005$
1 μM Colchicine	63.2 ± 26.0	−34.6 ± 26.9	$p < 0.005$
10 μM Colchicine	63.7 ± 23.1	−34.2 ± 23.8	$p < 0.005$
100 μM Colchicine	90.6 ± 8.6	−6.3 ± 8.9	$p < 0.1$

Mean ± SD, 20–25 cells per sample.

also examined changes in intracellular F-actin, a major component of the cytoskeleton. F-actin was labeled with BODIPY FL conjugated to phallacidin, a mushroom toxin which is known to bind specifically to the filamentous form of actin (De La Cruz and Pollard, 1994). Shown in Fig. 4 are fluorescent micrographs of cellular F-actin distribution in normal, paclitaxel-treated, and colchicine-treated cells. Consistent with previous reports (Sheterline and Rickard, 1989; Stossel, 1992), F-actin in normal neutrophils was mainly localized in the periphery of the cytoplasm, forming a "cortex" beneath the lipid membrane. Furthermore, the fluorescence was distributed fairly uniformly within the actin-rich cortex (Fig. 4 *a*). No difference in the pattern of the intracellular F-actin distribution was observed in cells treated with paclitaxel (Fig. 4 *b*). In contrast, colchicine induced dose-dependent changes in the ultrastructure of the F-actin network (Fig. 4, *c–f*). In cells treated with 0.1 μM colchicine, no obvious changes in the microfilament system were observed (Fig. 4 *c*). In the presence of 1 μM colchicine, most of the cells maintained the same pattern of F-actin distribution as normal cells, but disturbances in the F-actin network were visible in a very small number of cells (Fig. 4 *d*), which also exhibited minor irregularities in morphology. The alterations in the distribution of intracellular F-actin became more apparent as the dose of the drug was increased. This is evident from both the elevation of fluorescence intensity for F-actin and the localization of the microfilaments within the protrusions of the pseudopodia (Fig. 4, *e* and *f*).

Changes in the content of neutrophil intracellular (total) F-actin were characterized by flow cytometry. Shown in Fig. 5 are typical histograms of intracellular F-actin for normal cells and cells treated with paclitaxel or colchicine. Also shown (Fig. 5 *a, inset*) is a comparison between fluorescence distribution in normal cells incubated with 165 nM BODIPY FL-phallacidin (positive control) and cells incubated in PBS buffer alone (negative control). The negative control cells exhibited very low values of fluorescence ($<$100; *filled black area*) compared to positive control cells (500–700; *solid line*). Consistent with what was observed in

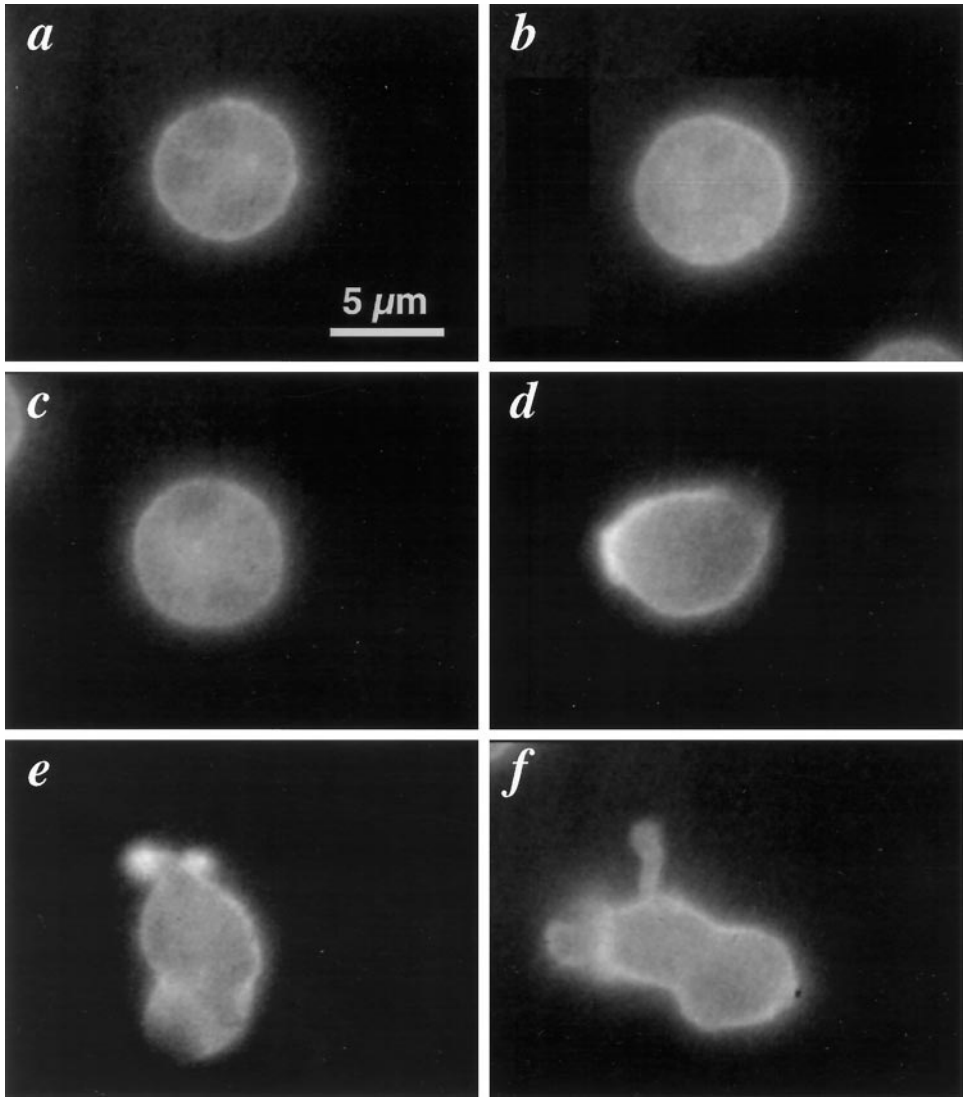

FIGURE 4 Effects of paclitaxel and colchicine on the distribution of neutrophil intracellular F-actin observed with epi-fluorescence microscopy. (*a*) Normal neutrophil; (*b*) cell treated with 2.5 μM paclitaxel; (*c–f*) cells treated with 0.1, 1, 10, and 100 μM colchicine, respectively. It is evident that paclitaxel has no significant effect on F-actin distribution, whereas colchicine causes actin polymerization and activates cells at concentrations of 10 μM or higher.

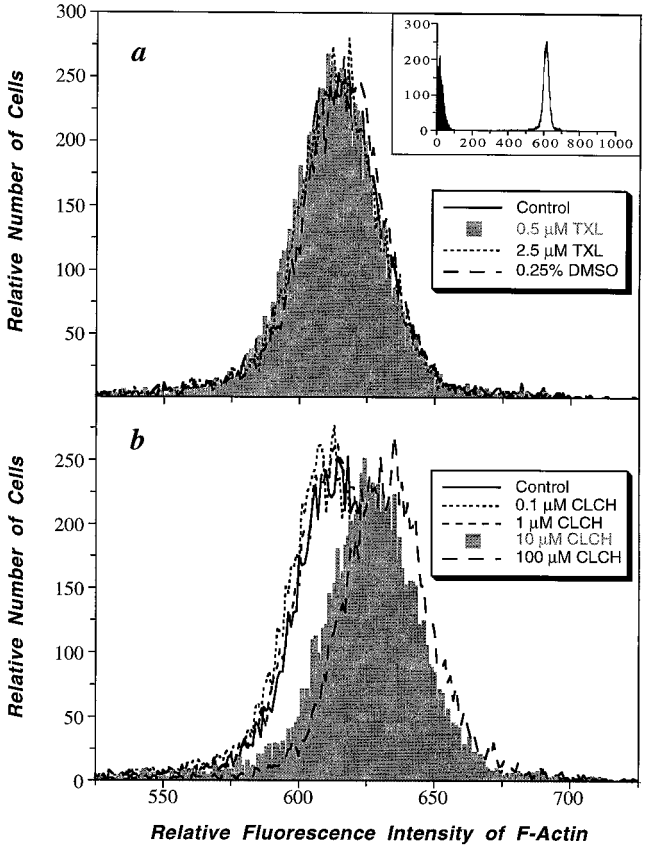

FIGURE 5 Effect of paclitaxel and colchicine on neutrophil intracellular F-actin content measured by flow cytometry. The insets are fluorescence distributions of positive (*solid line*) and negative (*filled area*) control. It is clear that 0.5–2.5 μM paclitaxel or 0.25% DMSO had no apparent effect on neutrophil intracellular F-actin (*a*). In contrast, treatment with 10 and 100 μM colchicine significantly increased neutrophil intracellular F-actin, whereas 0.1 or 1 μM colchicine had a very limited effect (*b*).

mechanical and morphological experiments, treatment with 0.5–2.5 μM paclitaxel or with its carrier solvent dimethyl sulfoxide (DMSO) had no effect on neutrophil microfilaments, and the cellular F-actin distributions in cells treated with paclitaxel and DMSO were almost identical to those of normal neutrophils. However, colchicine induced dramatic changes in F-actin content in a dose-dependent fashion. As shown in Fig. 5 *b*, the histogram of the intracellular F-actin distribution for cells treated with 10 μM colchicine was significantly shifted to the right (higher) relative to that of control cells, and the histogram for 100 μM colchicine-treated cells was further displaced to the right. Based on the average of six replicate experiments, 10 or 100 μM colchicine raised the content of intracellular F-actin by ~17% and 24%, whereas the drug at a concentration below 1 μM had a minimal effect.

In micromechanical experiments, only spherical neutrophils were selected for rheological measurements. To determine whether this selected cell subpopulation followed the overall population trend with regard to actin polymerization, individual spherical cells were selected under (red light) bright field and the cellular F-actin content was measured on a single-cell basis with epi-fluorescence microscopy. The results of these single-cell measurements were in good agreement with flow cytometric analysis. The mean fluorescence intensity of cells treated with DMSO alone, 0.5 μM or 2.5 μM paclitaxel, and 0.1 or 1 μM colchicine was within 4% of the intensity of untreated (control) cells. However, higher concentrations of colchicine (10 μM and 100 μM) caused a significant increase in actin polymerization, even though the morphology of the selected cells was spherical. Based on the single-cell measurements, 10 and 100 μM colchicine increased the content of intracellular F-actin in the subpopulation of spherical cells by ~25% and 28%, respectively.

DISCUSSION

Biological effects of colchicine and paclitaxel on microtubules

In the present study we used two well-studied microtubule reagents, paclitaxel and colchicine, to assess the specific role that microtubules may play in determining mechanical properties of neutrophils. Paclitaxel (Taxol) is an extract from the pacific yew tree, *Taxus brevifolia*, and has received attention as a new weapon against cancer. Its pharmacological effect has been extensively studied both in vivo and in vitro. It promotes tubulin polymerization and stabilizes microtubules from disassembly, thereby resulting in a net increase in cytoskeletal microtubules (Schiff et al., 1979; Manfredi et al., 1982; Rowinsky et al., 1988; Derry et al., 1995). As a consequence, proliferating cells are arrested in the G_2/M phase of the cell cycle and induced to undergo apoptosis in the presence of 10 nM to 10 μM paclitaxel (Schiff and Horwitz, 1980; Jordan et al., 1996; Milross et al., 1996). Immunofluorescence reveals that paclitaxel induces microtubules to form "asters" and "bundles" in these cultured cells (Roberts et al., 1989). Consistent with these reports, we observed that paclitaxel at 0.5–2.5 μM induced tubulin polymerization and formation of fluorescent-intense "hot spots" in neutrophils.

Colchicine is another well-known microtubule reagent that exerts an effect on microtubules opposite to the effect of paclitaxel. It binds to the β subunit of the tubulin heterodimer, forming a tubulin-colchicine complex and inhibiting tubulin polymerization into microtubules (Mareel and De Mets, 1984). Because of this inhibitory effect, cells cultured in the presence of colchicine are prevented from forming mitotic spindles and entering mitosis (Rieder and Palazzo, 1992). Based on indirect immunofluorescence microscopy, colchicine at 0.1 μM depolymerizes 90% of the microtubules in polymorphonuclear leukocytes; at a higher concentration (1 μM), it depolymerizes >95% of the microtubules in the cells (Anderson et al., 1982). In our own experiments, the fluorescence of tubulin in neutrophils treated with colchicine at concentrations as low as 0.1 μM appeared diffusive, and the fluorescence intensity was sig-

nificantly lower than normal cells (Fig. 3, *e–h*; Table 1), confirming the expected action of colchicine on microtubules. By contrasting the effects of these two microtubule reagents, the specific role of the cytoskeletal microtubules in determining neutrophil rheological properties can be elucidated.

In our previous studies we have shown that DMSO at a concentration up to 0.7% has no significant effect on leukocyte cortical tension and cellular viscosity (Tsai et al., 1993, 1996b). (In the present study, 0.5 and 2.5 μM paclitaxel solutions contained 0.05% and 0.25% DMSO, respectively.) Control experiments were also performed in this study to further exclude the possibility that DMSO may have nonspecific effects on microtubules or the microfilaments. Our results ensure that DMSO up to 0.25% does not alter the ultrastructure of microtubules, nor does it affect intracellular F-actin. Therefore, changes in cellular properties observed in this study are specifically related to the changes in the cytoskeleton.

Role of microtubules in leukocyte mechanical properties

The data presented in this study reveal that enhancement of the tubulin polymerization and stabilization of cytoskeletal microtubules by the reagent paclitaxel at a range of concentrations known to arrest cell growth in culture did not increase overall mechanical stability of the cells in terms of cell cortical tension and cellular viscosity, as assessed by micropipette aspiration. Surprisingly, treatment with high concentrations of the reagent colchicine, which is known to disrupt microtubules, did not soften the cells, but instead significantly rigidified the cells. In the range of concentrations (0.1–1 μM) at which 90–95% of cellular microtubules are depolymerized (Anderson et al., 1982), no changes in cellular mechanical properties were detected, and only at higher concentrations (10–100 μM) at which neutrophils became activated were changes observed. Therefore, alteration of the cytoskeletal microtubules by itself does not seem to have any direct effect on the mechanical properties of human neutrophils. This conclusion is consistent with the earlier findings from immunofluorescence microscopy and assays of neutrophil function. As is the case for most cells, microtubules in neutrophils are relatively few in number in comparison to the actin filaments (Anderson et al., 1982; Atkins and Anderson, 1982). Microtubule-depolymerizing reagents, e.g., colchicine, vinblastine, and vincristine, do not have the profound inhibitory effects on neutrophil mechanical function, in sharp contrast to the F-actin-specific toxins, the cytochalasins (Keller et al., 1984). Although the increase in intracellular F-actin caused by colchicine was moderate ($<$30%), changes in cellular deformability were dramatic. The characteristic viscosity increased by more than 60%, and when compared at the same aspiration pressures, neutrophil flow resistance more than doubled. In contrast, the promoter for microtubule assembly paclitaxel had no effect on the microfilaments, and neither did it cause any changes in the mechanical properties of the cells. These results further demonstrate that the actin filaments play the predominant role in determining the rheological properties of human neutrophils and that microtubules make a very limited contribution (Tsai et al., 1994, 1996a).

Previous reports have indicated that colchicine decreases the mechanical stiffness of neutrophils (Chien and Sung, 1984). The reason for the discrepancy between their results and ours is not entirely clear. The concentrations of colchicine used in those studies were 40–150 μM, overlapping the concentrations used in the present study, and the time of treatment was also similar. The differences may arise in part from differences in the way in which the mechanical properties were tested. In the former study, small pipettes and aspiration pressures were used to produce small, localized deformations of the cell surface, providing a test of the local viscoelasticity of the cell membrane and subjacent structural elements. The whole cell aspiration approach used in the present study provides an assessment of the viscous characteristics of the entire cytoplasm. It is unlikely that the decrease in cell rigidity the authors observed is due to the changes in cell cortical tension. We actually observed an increase in cell cortical tension when the cells were treated with 10–100 μM colchicine. One important difference in the experimental conditions was that the suspending buffer for the cells in the former study contained a calcium chelator (EDTA). In our hands, chelation of calcium results in a significant rigidification of the cells (unpublished observations). Thus, the apparent decrease in stiffness observed previously could have been the result of an antagonistic effect of colchicine and cellular rigidification caused by the removal of calcium. Activation of the cells by colchicine might also have contributed to the different results. (Activation of the cells by colchicine was observed in both studies.) Cell activation might lead to a greater ease of formation of localized protrusions, while simultaneously increasing the rigidity of the cells as a whole.

Much has been made in recent years of the possibility that cell mechanical behavior may arise from "tensegrity." In this conceptual model, it is proposed that contractile elements within the cell exist in balance with stiff, compression-resisting elements. In particular it has been proposed that contractile actin filaments may act in concert with compression-resistant microtubules to regulate cell shape and mechanical behavior (Ingber, 1993). The present results show clearly that this model is not applicable to neutrophils. Under conditions in which there is clear evidence of alterations in microtubular structure, no changes in cell shape or mechanical rigidity are evident. Only under conditions in which there is evidence of cell activation and accompanying changes in the presence and organization of actin filaments are alterations in mechanical properties detectable. These findings contradict expectations based on the tensegrity model that removal of the supporting microtubules should increase the net contractile forces on the cell interior imposed by the peripheral actin cytoskeleton. That no change

in cortical tension was observed under conditions in which the microtubule structure was substantially disrupted leads to the conclusion that microtubules play a negligible role in counteracting contraction of the actin cortex. Rather, our results support the view that cell properties are largely controlled by the state of the actin-based cytoskeleton, and that the contractile forces of the actin cortex are supported simply by the incompressibility of the cell interior and the regulation of cell volume by transport proteins in the plasma membrane.

CONCLUSION

Changes in microtubule structure caused by the reagents paclitaxel or colchicine have little or no effect on the rheological behavior of neutrophil cytosol. However, a secondary effect of colchicine treatment at a concentration of 10 μM or higher results in increased levels of F-actin within cells, changes in cell morphology, and a decrease in cell deformability. These results further support the view that the mechanical properties of human neutrophils are predominately regulated by actin microfilaments.

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