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Cytoplasmic Microtubules in Human Neutrophil Degranulation: Reversible Inhibition by the Colchicine Analogue 2-Methoxy-5-(2′,3′,4′-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one

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SUMMARY

The colchicine analogue 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one (MTC) was found to inhibit concanavalin A- and formyl-methionyl-leucyl-phenylalanine-stimulated human neutrophil degranulation and to depolymerize the microtubule network at low concentrations. The inhibitory capacity of MTC for neutrophil degranulation was similar to that of nocodazole and taxol. The mechanistically distinct actions of these three drugs on microtubules support the notion that microtubules are required for neutrophil enzyme release in response to different stimuli. MTC affected both degranulation and microtubule integrity rapidly and reversibly, after only a 5-min preincubation. At these short periods of incubation, colchicine irreversibly affected neutrophil degranulation only at concentrations in the millimolar range and behaved similarly to its microtubule-inactive analogue lumicolchicine. At longer times of incubation (30–60 min), low concentrations of both MTC and colchicine induced a drastic shortening and depolymerization of microtubules, preserving the microtubule-organizing center, but only MTC was able to completely inhibit the secretory response of neutrophils. These results suggest that the colchicine effect on neutrophil degranulation is not specifically mediated by its action on the microtubule network of these cells. In contrast, the specific and reversible effects of the colchicine analogue MTC suggest that it may be a useful agent with which to study the role of microtubules in this cellular function.

Central to the function of neutrophils are the substances that are stored in the cytoplasmic granules. Two main types of granules (azurophilic and specific granules), differing in size, mass, and biochemical composition, have been identified in human neutrophils (1). Recent evidence reveals the presence in human neutrophils of an additional third population of intracellular granules, not yet well characterized (2, 3). Upon cell activation, there is a sequential and highly regulated movement of these intracellular organelles towards the plasma membrane or the endocytic vacuole (4). Fusion of the intracellular granules with the cell surface (secretion) or with the endocytic vacuole (phagocytosis) is essential for the inflammatory and microbicidal actions of human neutrophils. However, the molecular mechanisms underlying these membrane fusion processes remain to be elucidated.

Microtubules are considered to be of pivotal importance in stabilizing cellular shape and in controlling cellular motility and intracellular granule flow (5–9). However, the precise relationship between microtubules and neutrophil degranulation remains unclear. This is due, in part, to the uncertain specificity of the cytoskeleton inhibitors used in most studies. Colchicine has been widely reported to inhibit enzyme release in human neutrophils stimulated by several particulate or soluble stimuli (10–12), but it also has been found to be ineffective in inhibiting secretion induced by the calcium ionophore A23187 (13). The reported inhibitory effect of colchicine on degranulation may be attributed to effects other than microtubule disruption, because the concentration of this agent required for inhibition of enzyme release was substantially greater than that usually needed for disruption of microtubules. Furthermore, the absence of microtubule-inactive analogue controls, such as lumicolchicine, in these studies precluded a conclusive statement. Also, it has been shown that microtubule assembly can be partially dissociated from granule enzyme secretion by appropriate pharmacological manipulation, suggesting a role for other cytoskeletal elements (14).

In the present paper, we have studied the effect of colchicine

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ABBREVIATIONS: MTC, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; Con A, concanavalin A, DMSO, dimethylsulfoxide; FMLP, formyl-methionyl-leucyl-phenylalanine; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; MTOC, microtubule-organizing center; MTPC, 2-methoxy-5-(3-(3,4,5-trimethoxyphenyl)propionyl)amino)-2,4,6-cycloheptatrien-1-one; PIPES, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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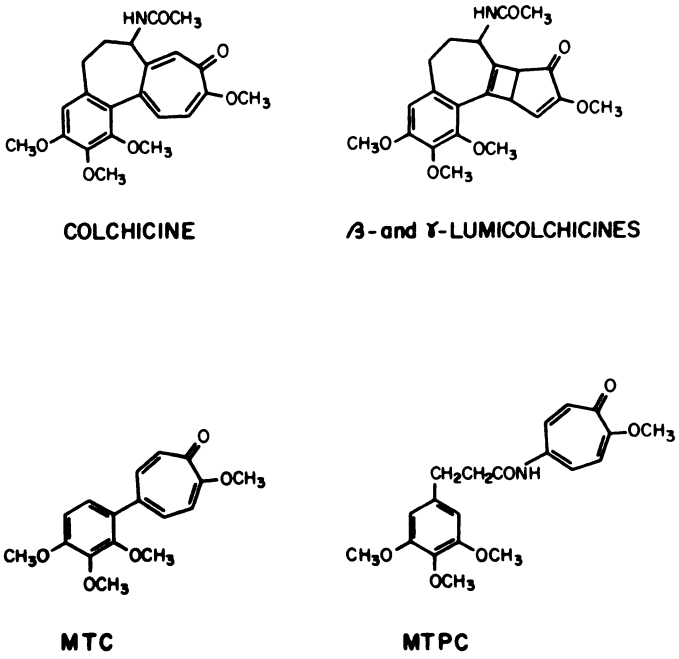

Fig. 1. Structures of colchicine, lumicolchine, MTC, and MTPC.

and MTC, a newly employed bicyclic colchicine analogue (15), on human neutrophil degranulation and on the microtubule network of these cells studied by indirect immunofluorescence. Fig. 1 shows the structure of these compounds. MTC is a microtubule inhibitor missing the middle ring of colchicine (15). This compound contains the two essential parts of colchicine that are required for binding to the tubulin site (16), namely the trimethoxyphenyl and the tropolone methyl ether rings of the colchicine molecule. The crystal conformation of MTC is similar to that of colchicine (17). MTC binds rapidly and reversibly to the high affinity colchicine binding site of the tubulin molecule and inhibits microtubule assembly *in vitro* substoichiometrically (18–20). We have compared the effects of MTC on neutrophil degranulation with those exerted by other microtubule-affecting compounds, by immunofluorescence and biochemical experiments. Appropriate control experiments were carried out with the microtubule-inactive analogues of colchicine and MTC, lumicolchicine and MTPC, respectively (Fig. 1). When colchicine is irradiated with long wavelength UV light, it is converted into a mixture of the steroisomers β- and γ-lumicolchicine (21). Colchicine and lumicolchicine are structurally similar (see Fig. 1) and both agents interact with the plasma membrane to inhibit the transport of nucleosides to the same degree, whereas lumicolchicine does not disrupt microtubules (22, 23). The compound MTPC binds weakly to tubulin and affects microtubule assembly only at high concentrations, and it can be considered as a conformationally inactive analogue of MTC (18).

Materials and Methods

Drugs. Stock solutions of 0.1 M MTC (kindly provided by Dr. T. J. Fitzgerald, Florida A & M University) (15), 0.1 M MTPC (kindly provided by Dr. M. Gorbunoff, Brandeis University) (18), 0.01 M nocodazole (Sigma, St. Louis, MO), and 0.01 M taxol (kindly provided by Dr. M. Suffness, National Cancer Institute, National Institutes of Health) in DMSO were used in these experiments and were stored at −20°. Stock solutions of 0.1 M colchicine (Aldrich Chemical Co., Steinheim FRG) and 0.1 M lumicolchicine in H_2O were stored at −20°. Lumicolchicine was prepared by long wavelength UV irradiation of colchicine (21). The reaction was followed spectrophotometrically and continued for eight half-lives and the product contained no detectable colchicine by thin layer chromatography. For cell treatments, the various drugs were diluted in HEPES buffer (150 mM NaCl, 5 mM KOH, 10 mM HEPES, 1.2 mM $MgCl_2$, and 1.3 mM $CaCl_2$, 5.5 mM glucose, pH 7.5) to the final concentrations indicated in the respective legends to the figures. DMSO did not affect the assays at the concentrations used (less than 1%).

Isolation of neutrophils. Neutrophils were obtained from fresh human peripheral blood as described (24), with slight modification of the procedure. Leukocyte suspensions were prepared after sedimentation of erythrocytes in 1.3% dextran (Sigma) at room temperature. The leukocyte-rich supernate was centrifuged at 300 × *g* for 10 min and the pellet was gently resuspended in 20 ml of phosphate-buffered saline solution, loaded onto a layer of 20 ml of Lymphoprep (Nyegaard, Oslo, Norway), and centrifuged at 400 × *g* for 40 min at room temperature to separate mononuclear cells from granulocytes. The neutrophil-enriched pellet was resuspended in 8 ml of phosphate-buffered saline and contaminating erythrocytes were hypotonically lysed by addition of 24 ml of distilled water for about 30 sec. Isotonicity was then immediately restored by addition of 8 ml of 3.6% (w/v) NaCl and neutrophils were pelleted by centrifugation at 300 × *g* for 10 min. The lysis/wash step was repeated, if necessary, to get a final neutrophil suspension that was free of erythrocytes. The final cell preparation contained more than 98% neutrophils, as assessed by Giemsa-Wright stain. Cell viability was evaluated by trypan blue dye exclusion and was always higher than 98%.

Granule enzyme release. Aliquots (1 ml) of freshly isolated neutrophils (10×10^6), resuspended in HEPES buffer, were preincubated with 5 μg/ml cytochalasin B (Sigma) for 5 min at 37°, to optimize enzyme release by 'reverse endocytosis' (25, 26), in the presence and absence of microtubule-active drugs. Cells were then incubated at 37° for an additional 5 min in either the absence (control) or the presence of various stimuli shown in the respective figures. Incubations were terminated by centrifugation for 10 min at 300 × *g* at 4°. The cellular supernatants were assayed for the granule enzyme markers β-glucuronidase (azurophilic granules) and lysozyme (specific and azurophilic granules), as well as for the cytosolic enzyme lactate dehydrogenase, as previously described (3). Lysozyme is localized both in specific (about two thirds of the total cell lysozyme) and in azurophilic (about one third of the total cell lysozyme) granules (27) but it can be used as a reliable marker to indicate release of specific granule contents when it is assayed in parallel with a specific marker for azurophilic granules, such as β-glucuronidase. In order to preferentially stimulate the secretion of the specific granules, we used 100 μg/ml Con A (28). To stimulate both azurophilic and specific granules, we used 10^{-7} M FMLP (29). The total enzyme content of these cells was measured in aliquots after cellular disruption by addition of 0.2% (v/v) Triton X-100. The nontoxicity of the agents was confirmed by the trypan blue exclusion test, which showed over 96% viability of the cells in the presence of the drugs under the experimental conditions used. Also, determinations of lactate dehydrogenase in all the experiments resulted in less than 6% enzyme release to the extracellular medium, indicating good cell viability. Enzyme release is expressed, unless otherwise indicated, as percentage of the enzyme activity released by the stimulated cells in the absence of any microtubule-active agent.

For recovery experiments, cells were preincubated in the presence of the MTC or colchicine for 5 min at 37°, as described above, followed by removal of the microtubule-active drugs and three washes in HEPES buffer. Cells were then stimulated as indicated in the respective table and compared with the stimulation of untreated cells.

Unless otherwise indicated, the results given are the mean (± standard error) of the number of experiments indicated.

Immunofluorescence. Neutrophils were treated with several microtubule-active drugs, as described above, and were subsequently

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examined by immunofluorescence after the cells were diluted from 10^7 cells/ml to 2×10^5 cells/ml in HEPES buffer, in the presence of the drugs employed. One hundred microliters of the cell suspension were allowed to attach to 9 × 9 mm glass coverslips for 5 min at 37°. The various drug treatments employed did not significantly modify neutrophil adhesion to the glass. Coverslips were washed eight times in PEM (100 mM PIPES, 1 mM EGTA, 2 mM $MgCl_2$, pH 6.8) containing 4% (w/v) polyethyleneglycol 8000 and were incubated for 90 sec with PEM containing 0.5% (w/v) Triton X-100 to permeabilize the cells. After extensive rinsing (eight times) with PEM containing 4% (w/v) polyethylene glycol, cells were fixed with 3.7% formaldehyde in PEM containing 1% DMSO, for 30 min at room temperature, and were finally washed in phosphate-buffered saline (30). Then, the coverslips were incubated for 60 min at 37° in a humidified Petri dish with antitubulin antibodies. The antibodies used in this study were mouse monoclonal anti-α and anti-β tubulin antibodies (Amersham, Buckinghamshire, UK) that were diluted 1:400 in phosphate-buffered saline or rabbit polyclonal antibodies raised against tubulin synthetic peptides (31) that were diluted 1:50. After being washed twice with phosphate-buffered saline (10 min each wash) with gentle shaking, the coverslips were incubated for 45 min with fluorescein-labeled goat anti-mouse antibodies (Amersham) or fluorescein-labeled goat anti-rabbit antibodies (Behring, FRG) that were diluted 1:20 in phosphate-buffered saline, washed twice with phosphate-buffered saline (10 min each wash), mounted with 90% glycerol antifading solution (32), and stored in the dark at −20° until observation.

For assays of the microtubular network recovery, neutrophils were allowed to attach to the coverslips as described above, in the presence of the microtubule-active drugs, and then they were rinsed with HEPES buffer in the absence of any drug, incubated for the time indicated in the same buffer, and subsequently fixed and processed for immunofluorescence as described above.

The coverslips were examined with a Zeiss microscope equipped with epifluorescence illumination, using a Zeiss Planapochromatic 63× oil-immersion objective. Pictures were taken on Kodak Tri-X film. Negative controls were routinely prepared by omitting the first antibody. These latter typically showed a weak granular fluorescence but no filamentous structures.

Results

Effect of MTC and colchicine on neutrophil degranulation. We have measured the release of specific and azurophilic granule contents in cytochalasin B-treated human neutrophils. We have employed cytochalasin B-treated cells in order to be able to monitor extracellularly (after fusion of cytoplasmic granules with plasma membrane) the processes that ordinarily occur intracellularly (fusion of cytoplasmic granules with endocytic vacuoles) and to obtain a higher secretory response upon cell stimulation. Cytochalasin B interferes with the function of cytoplasmic microfilaments, inhibiting the ingestion of particles, facilitating the fusion of intracellular granules with the cell surface (26), and affecting the down-regulation of receptor activity by inhibiting receptor-cytoskeleton interaction (33). Figs. 2 and 3 show that both colchicine and its analogue MTC inhibit the enzyme release upon neutrophil stimulation by 10^{-7} M FMLP, in a concentration-dependent fashion. FMLP is a complete secretagogue, inducing the discharge of both specific and azurophilic granules (29). The inhibition of lysozyme release suggested an effect on the mobilization of specific granules. To check this, we stimulated neutrophils with 100 μg/ml Con A, which induces the discharge only of specific granules in neutrophils (28) and did not induce β-glucuronidase release under our conditions. Fig. 4 shows that both colchicine and its analogue MTC inhibit degranulation of

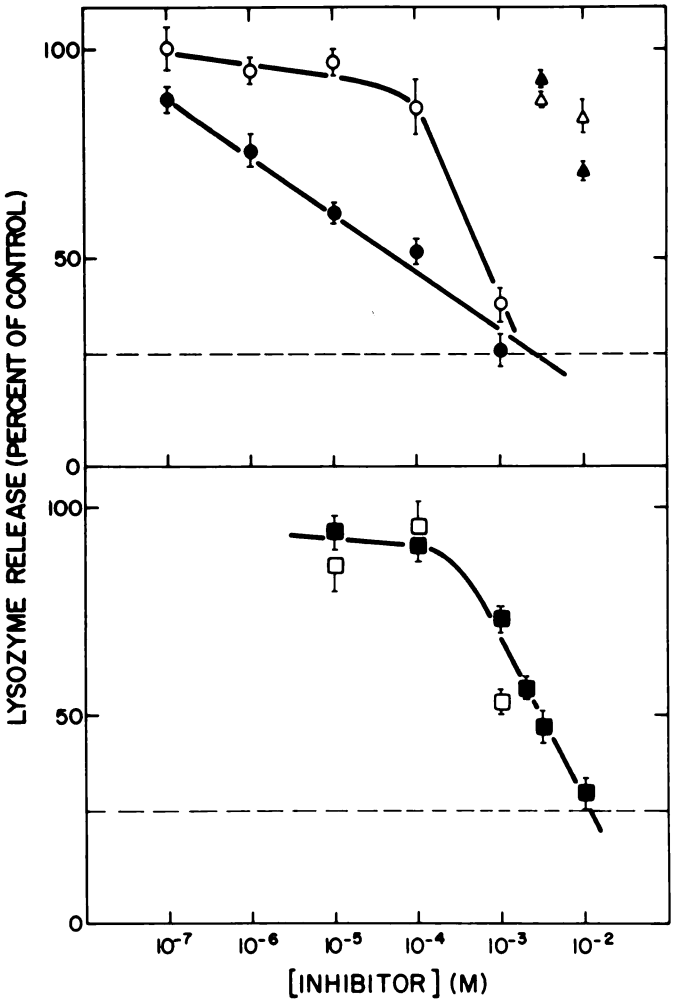

Fig. 2. Effect of different microtubule-active agents on degranulation of specific granules in cytochalasin B-treated human neutrophils stimulated by FMLP. Neutrophils (10×10^6) were incubated at 37° for 5 min with cytochalasin B (5 μg/ml), in the presence and in the absence of the compounds listed below. Then, cells were incubated at 37° for an additional 5 min in either the presence or absence of 10^{-7} M FMLP as stimulus. Subsequently, cells were pelleted by centrifugation and lysozyme was assayed in the supernatants. Percentage of control enzyme release is plotted as a function of MTC (●), MTPC (○), *N*-acetylmescaline (▲), tropolone methyl ether (△), colchicine (■), and lumicolchicine (□) concentrations. Mean values ± standard errors of at least five independent experiments are represented. Control cells preincubated with cytochalasin B, but in the absence of any microtubule-disrupting drug, released 36.3 ± 4.0% of the total cellular lysozyme activity when activated with 10^{-7} M FMLP, representing 100% of enzyme release. In the absence of stimulus, 9.8 ± 1.2% of the total cellular lysozyme activity was released, representing 27.0% of control enzyme release (*dashed line*).

specific granules. After only a 5-min preincubation with cells, MTC was able to inhibit enzyme release from both types of granules at low concentrations, whereas colchicine was only effective at the millimolar range. As shown in Table 1, MTC was 20 to 100 times more effective than colchicine. Interestingly, azurophilic granule release was consistently more sensitive to MTC than specific granule release, by approximately 1 order of magnitude (Table 1).

Control experiments with the inactive analogues of colchicine and MTC, lumicolchicine and MTPC, respectively, are shown in Figs. 2 and 3. The parallel effects displayed by colchicine and lumicolchicine indicate that inhibition of neutrophil de-

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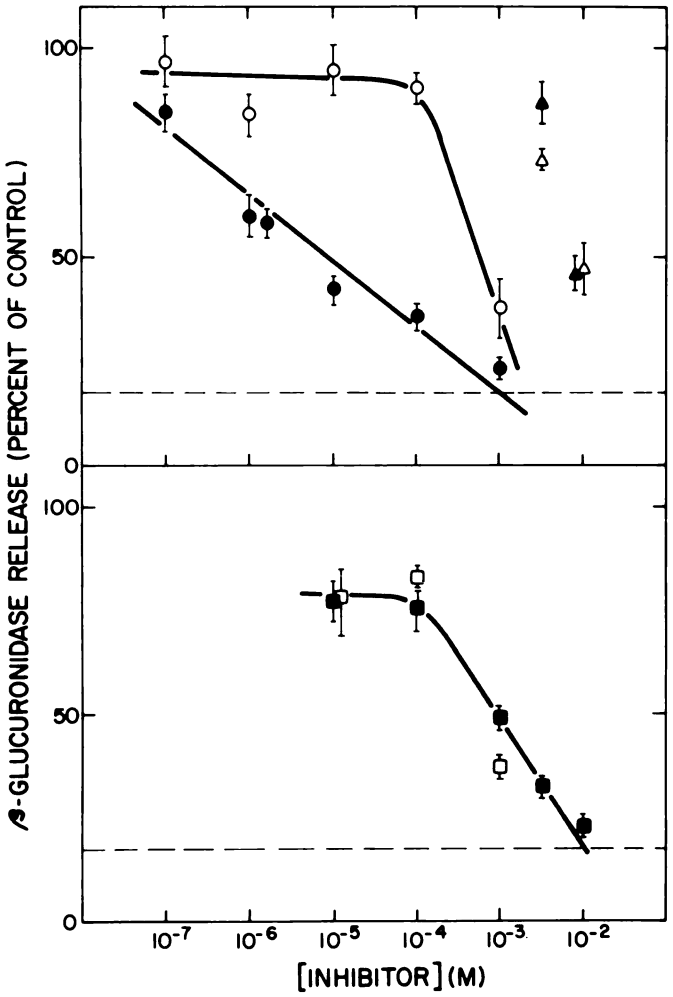

Fig. 3. Effect of different microtubule-active agents on degranulation of azurophilic granules in cytochalasin B-treated human neutrophils stimulated by FMLP. Neutrophils (10×10^6) were incubated at 37° for 5 min with cytochalasin B (5 μg/ml) in the presence and in the absence of the compounds listed below. Then, cells were incubated at 37° for an additional 5 min in either the presence or absence of 10^{-7} M FMLP as stimulus. Subsequently, cells were pelleted by centrifugation and β-glucuronidase was assayed in the supernatants. Percentage of control enzyme release is plotted as a function of MTC (●), MTPC (○), *N*-acetylmescaline (▲), tropolone methyl ether (△), colchicine (■), and lumicolchicine (□) concentrations. Mean values ± standard errors of at least five independent experiments are represented. Control cells preincubated with cytochalasin B, but in the absence of any microtubule-disrupting drug, released 22.1 ± 1.9% of the total cellular β-glucuronidase activity when activated by 10^{-7} M FMLP, representing 100% of enzyme release. In the absence of stimulus, 3.8 ± 0.6% of the total cellular enzyme activity was released, representing 17.2% of control enzyme release (*dashed line*).

granulation by colchicine is not due only to microtubule disruption but also to another effect exerted by colchicine in the cell. In contrast, MTPC was only partially active in inhibiting neutrophil degranulation at concentration higher than 10^{-4} M, whereas MTC was effective even at 10^{-7} M. This suggests that inhibition of granule enzyme release in human neutrophils by low concentrations of MTC could be due to its direct action inhibiting tubulin polymerization. Interestingly, analogues of the two tubulin-binding parts of the MTC and colchicine molecule, namely tropolone methyl ether and *N*-acetylmescaline, were able to partially inhibit the enzyme granule release (Figs. 2 and 3), although at relatively high concentrations.

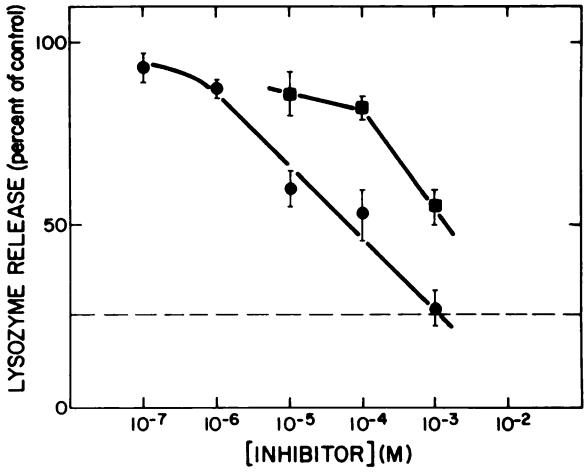

Fig. 4. Effect of colchicine and MTC on degranulation of specific granules in cytochalasin B-treated human neutrophils stimulated by Con A. Neutrophils (10×10^6) were incubated at 37° for 5 min with cytochalasin B (5 μg/ml) in the presence and in the absence of the microtubule-disrupting drugs. Then, cells were incubated at 37° for an additional 5 min in either the presence or absence of 100 μg/ml Con A as stimulus. Subsequently, cells were pelleted by centrifugation and lysozyme was assayed in the supernatants. Percentage of control enzyme release is plotted as a function of colchicine (■) and MTC (●) concentrations. Mean values ± standard errors of at least four independent experiments are represented. In the absence of any inhibitor (control) 29.6 ± 2.3% of the total cellular lysozyme activity was released by incubation of cells with Con A and represents 100% of enzyme release. In the absence of stimulus, 7.5 ± 0.5% of the total cellular lysozyme activity was released, representing 25.4% of control release (*dashed line*).

TABLE 1

Half-inhibitory MTC and colchicine concentrations for enzyme granule release in human neutrophils

Half-inhibitory concentrations were calculated from lines drawn in Figs. 2, 3, and 4. Neutrophils (10×10^6) were incubated at 37° for 5 min with cytochalasin B (5 μg/ml) in the presence or absence (controls) of MTC or colchicine. Then, cells were incubated at 37° for an additional 5 min in either the presence or absence of the stimuli indicated.

Enzyme marker	Stimulus	Half-inhibitory MTC concentration	Half-inhibitory colchicine concentration
		M	
Lysozyme	Con A (100 μg/ml)	8×10^{-5}	1.5×10^{-3}
Lysozyme	FMLP (10^{-7} M)	8×10^{-5}	4.5×10^{-3}
β-Glucuronidase	FMLP (10^{-7} M)	10^{-5}	10^{-3}

MTC was also active at low concentrations in inhibiting lysozyme release in neutrophils that were activated with the particulate stimulus zymosan, in the absence of cytochalasin B (results not shown).

Reversibility of the inhibitory effect of MTC on human neutrophil degranulation. The effect of MTC upon release of granule enzymes was completely reversible. Cells exposed to MTC for 5 min at 37° and then washed three times with buffer responded comparably to untreated cells, when subsequently challenged with 10^{-7} M FMLP (Table 2). In contrast, the effect of colchicine on granule enzyme release was poorly reversible under the experimental conditions used.

Time-course of MTC and colchicine effects on neutrophil degranulation. In order to test whether the greater effect of MTC, as compared with that of colchicine, could be due to kinetic effects, we studied the time-course of granule release inhibition by these two drugs at 10^{-5} M. As can be seen in Fig. 5, 10^{-5} M MTC totally inhibited enzyme release in cytochalasin B-treated neutrophils that were stimulated by FMLP after a

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TABLE 2

Reversibility of the MTC and colchicine effects on human neutrophil degranulation upon FMLP stimulation

Neutrophils (10×10^6) were incubated at 37° for 5 min with cytochalasin B (5 μg/ml) in the presence or in the absence ("None") of 10^{-4} M MTC or 10^{-3} M colchicine. Then, a portion of the cells were incubated at 37° for an additional 5 min in either the presence or the absence ("without stimulation") of 10^{-7} M FMLP, and a portion of the cells were washed three times with HEPES buffer and subsequently stimulated with 10^{-7} M FMLP as described above. Results are mean values ± standard errors of three independent experiments.

Drug	Lysozyme release		β-Glucuronidase release	
	Without washing	After three washes	Without washing	After three washes
	%			
None	100	100	100	100
MTC (10^{-4} M)	76.2 ± 3.1	112.7 ± 4.8	28.1 ± 3.3	108.7 ± 5.7
Colchicine (10^{-3} M)	87.8 ± 1.0	88.4 ± 2.1	44.2 ± 2.8	63.0 ± 5.8
Without stimulation	20.3 ± 0.5	19.6 ± 0.3	15.4 ± 1.1	14.5 ± 1.7

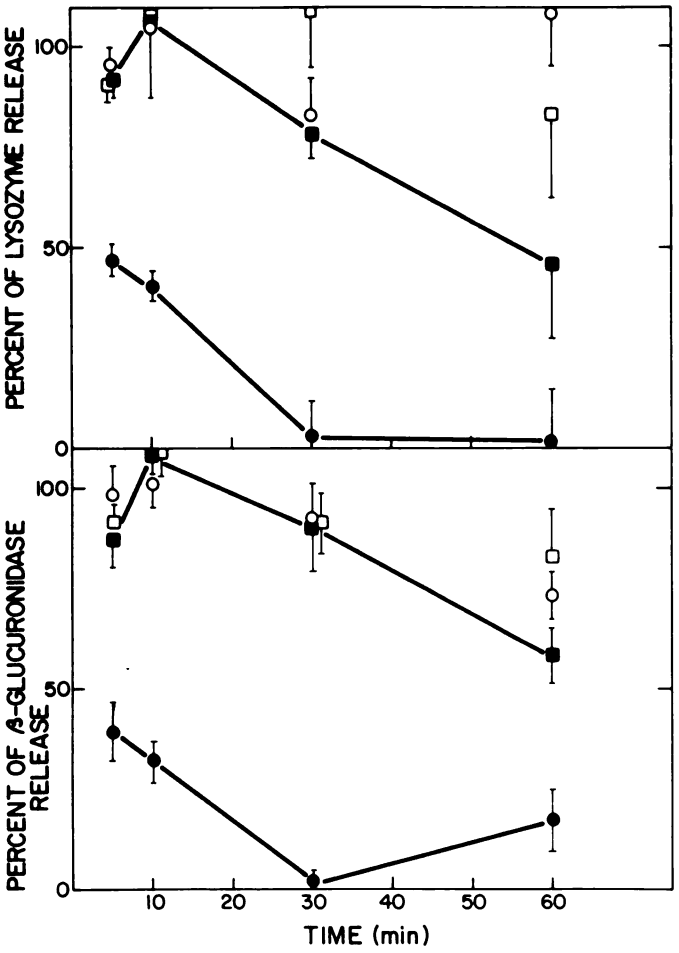

Fig. 5. Time course of the effect of MTC and colchicine on enzyme release from human neutrophils. Neutrophils (10×10^6) were preincubated with cytochalasin B (5 μg/ml) and with 10^{-5} M MTC (●), 10^{-5}M MTPC (○), 10^{-5} M colchicine (■), or 10^{-5} M lumicolchicine (□), at 37° for the time indicated, and then exposed to 10^{-7} M FMLP for 5 min at 37°. Enzyme activity released by the treated cells is shown as percentage of the enzyme activity released by controls. Neutrophils preincubated with cytochalasin B, but in the absence of any microtubule-disrupting drug, for the time indicated and then stimulated with 10^{-7} M FMLP were used as controls (100% enzyme release). Percentage of enzyme release was calculated as follows: % = (% enzyme release from stimulated drug-treated cells − % enzyme release from unstimulated cells/100 − % enzyme release from unstimulated cells) × 100. Lysozyme and β-glucuronidase release from controls were 36.3 and 22.1% (5 min), 40.5 and 20.2% (10 min), 44.3 and 35.2% (30 min), and 47.3 and 37.5% (60 min), respectively, of the total cellular enzyme activity. Mean values ± standard errors of three independent experiments are represented.

30-min preincubation of the cells with the drug at 37°, whereas 10^{-5} M colchicine produced only about 50% inhibition of the degranulation process after a 60-min preincubation. Preincubation with the respective inactive analogues, under identical experimental conditions, did not affect degranulation appreciably. The distinct degrees of enzyme release inhibition with increasing preincubation times shown by MTC and colchicine could reflect the different times required by both drugs to bind to tubulin. The slow binding of colchicine to tubulin could explain the increase of enzyme release inhibition at prolonged preincubation times.

Comparison of MTC with other microtubule-active agents on neutrophil enzyme release inhibition. Table 3 shows the comparative effects of MTC and other microtubule-active drugs on the enzyme release from cytochalasin B-treated FMLP-activated human neutrophils. MTC behaved similarly to nocodazole, a structurally unrelated drug that inhibits microtubules (34), and to taxol, an alkaloid that binds specifically to polymerized tubulin, leading to stabilization of existing microtubules and formation of aberrant microtubule networks (35, 36).

TABLE 3

Inhibition of enzyme release from cytochalasin B-treated human neutrophils exposed to FMLP by MTC, in comparison with other microtubule-active agents

Compound	Concentration of inhibitor added[a]	Enzyme release	
		Lysozyme	β-Glucuronidase
	M	% of control	
None		100.0	100.0
Nocodazole	10^{-7}	98.7 ± 12.2	86.0 ± 1.4
Nocodazole	10^{-5}	68.5 ± 4.0	54.6 ± 3.4
Nocodazole	10^{-4}	56.3 ± 10.5	49.5 ± 3.8
Taxol	5×10^{-5}	45.5 ± 4.5	36.9 ± 3.1
Taxol	10^{-4}	23.9 ± 3.9	24.1 ± 1.5
MTC	10^{-7}	87.9 ± 3.5	84.7 ± 4.6
MTC	10^{-5}	61.4 ± 2.1	42.5 ± 3.2
MTC	10^{-4}	52.1 ± 3.1	36.1 ± 3.3
		Enzyme release[b]	
		Lysozyme	β-Glucuronidase
		% of total	
Resting cells		9.8 ± 1.2	3.8 ± 0.6
Cells + 10^{-7} M FMLP		36.3 ± 4.0	22.1 ± 1.9

[a] Neutrophils (10×10^6) were preincubated with cytochalasin B (5 μg/ml) and the corresponding drug for 5 min at 37° and then exposed to 10^{-7} M FMLP for an additional 5 min at 37°, as decribed in Materials and Methods.

[b] Enzyme activity is expressed as a percentage of total cellular activity determined by cell disruption by repeated freezing/thawing in the presence of 0.2% Triton X-100. Results are mean values ± standard errors of three independent experiments.

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Effects of microtubule-active agents on the microtubule network of human neutrophils studied by immunofluorescence. The effects of MTC and other microtubule inhibitors on neutrophil cytoplasmic microtubules were monitored by indirect immunofluorescence in the presence and in the absence of cytochalasin B (Figs. 6 and 7). Resting neutrophils showed a relatively homogeneous population of microtubules, originating from a single MTOC. About 30 microtubules, each approximately 5–8 μm in length, can be counted in resting cells (Figs. 6a, 7a, and 8a). Microtubules appear to extend radially from the MTOC toward the plasma membrane, where they terminate or bend sharply. After a 5-min preincubation with 10^{-5} M MTC or colchicine a partial depolymerization of microtubules could be observed (results not shown), indicating that both drugs were able to get into the cells at this short preincubation time. After a 30-min preincubation, MTC at 10^{-5} M induced an almost total microtubule depolymerization, both in cytochalasin B-treated (Fig. 6c) and untreated (Fig. 7c) human neutrophils, even though the MTOC and a few short microtubules (1–3 μm in length) radiating from it were still visible. However, 10^{-5} M MTPC was unable to promote disassembly of microtubules (Figs. 6d and 7d). Likewise, colchicine at 10^{-5} M and after a 30-min preincubation induced microtubule disruption (Figs. 6e and 7e), whereas lumicolchicine at the same concentration was ineffective (Figs. 6f and 7f). These results broadly agree with the capacity of MTC and colchicine to inhibit the degranulation process. Nevertheless, the results do not explain the different capacities shown by MTC and colchicine in inhibiting the secretory process in human neutrophils. Unlike MTC, taxol induced an increase in tubulin polymerization (Figs. 6b and 7b), in agreement with the well known effect of this drug on microtubule assembly (35), indicating

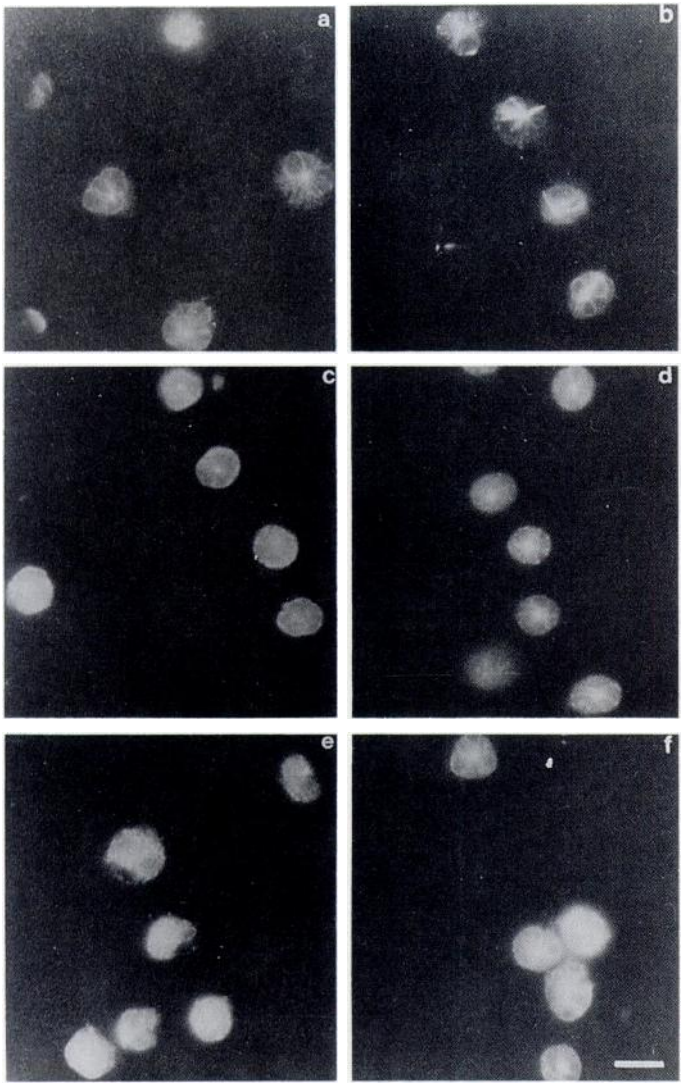

Fig. 6. Effects of MTC and other microtubule inhibitors on the cytoplasmic microtubule network in cytochalasin B-treated human neutrophils. Neutrophils were treated with the drug for 30 min at 37° in the presence of cytochalasin B (5 μg/ml) and were stained with tubulin antibodies as described in Materials and Methods. a, Untreated cells; b, cells after treatment with 10^{-5} M taxol; c, cells after treatment with 10^{-5} M MTC; d, cells after treatment with 10^{-5} M MTPC; e, cells after treatment with 10^{-5} M colchicine; f, cells after treatment with 10^{-5} M lumicolchicine. *Bar*, 10 μM.

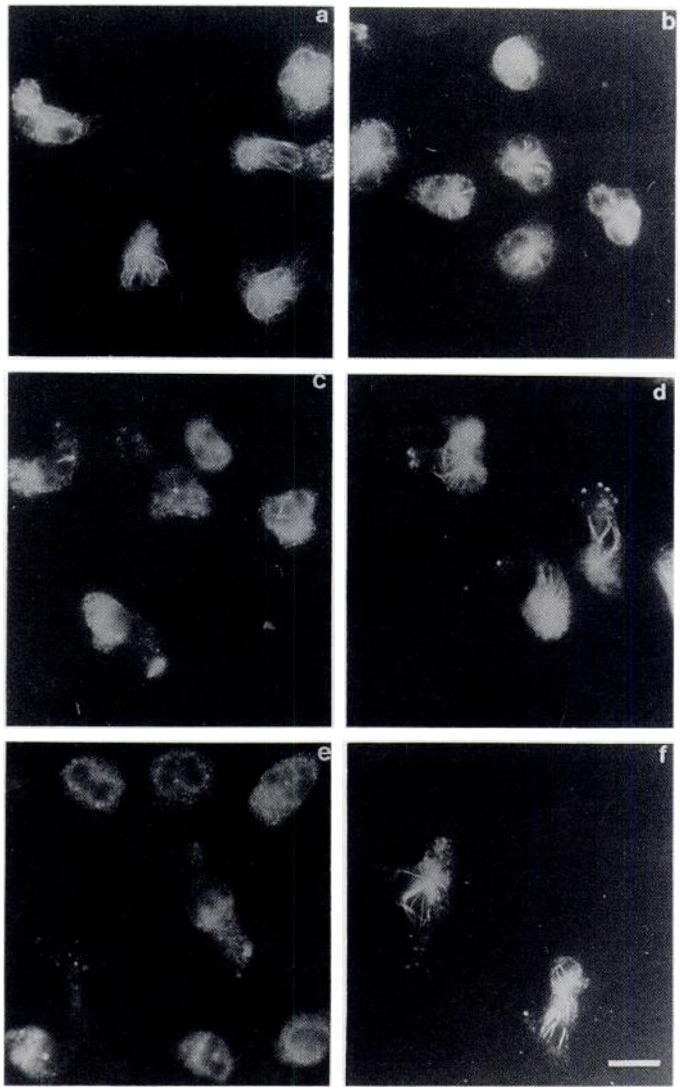

Fig. 7. Effects of MTC and other microtubule inhibitors on the cytoplasmic microtubule network in human neutrophils. Neutrophils were treated with drugs for 30 min at 37° in the absence of cytochalasin B and were stained with tubulin antibodies as described in Materials and Methods. a, Untreated cells; b, cells after treatment with 10^{-5} M taxol; c, cells after treatment with 10^{-5} M MTC; d, cells after treatment with 10^{-5} M MTPC; e, cells after treatment with 10^{-5} M colchicine; f, cells after treatment with 10^{-5} M lumicolchicine. *Bar*, 10 μm.

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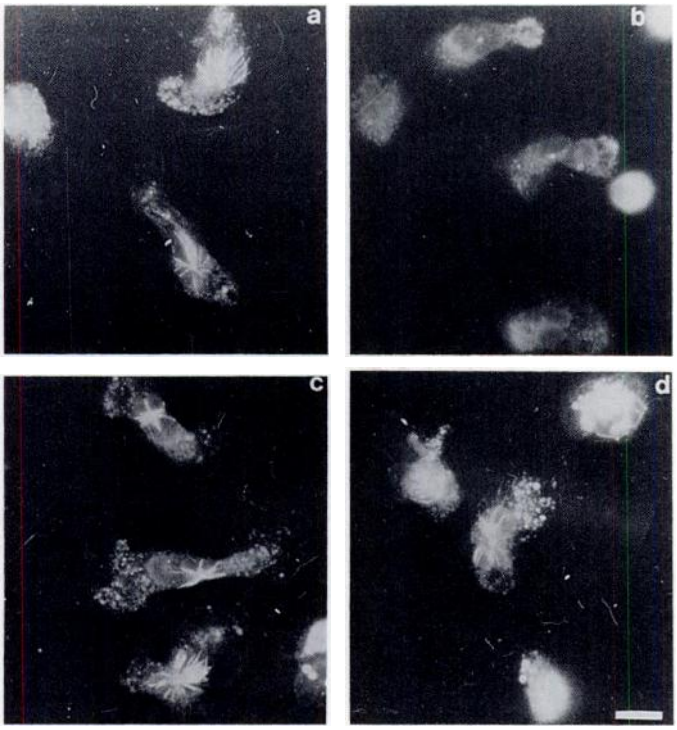

Fig. 8. Reassembly of the microtubule network after removal of MTC from the medium. Human neutrophils were treated with 10^{-5} M MTC for 30 min at 37° and, after removal of the drug for different times, the cells were stained by immunofluorescence as described in Materials and Methods. a, Untreated cells; b, cells treated with 10^{-5} M MTC for 30 min; c, MTC-treated cells after removal of the drug for 2 min; d, MTC-treated cells after removal of the drug for 5 min. *Bar* 10 μm.

that functional microtubules are required for a normal secretory response in human neutrophils.

On the other hand, it is noteworthy to observe that cytochalasin B treatment induced profound alterations in cell morphology (compare Figs. 6a and 7a). Cytochalasin B induced rounded cells and a poor visualization of the microtubule network, in agreement with the important role of the microfilaments in maintaining the cell shape (37, 38).

Reversibility of microtubule-affecting agents on the microtubule network. As shown in Fig. 8, MTC showed a clear-cut reversible effect on microtubule disruption. In MTC-treated cells, a shortening of the microtubule length was observed, maintaining a single MTOC. Regeneration of microtubules was observed shortly after removal of MTC, being complete after 5 min (Fig. 8d). Regeneration of individual microtubules occurred via centrifugal elongation from the MTOC. In contrast, colchicine behaved as a nonreversible microtubule-affecting agent in this time scale (results not shown).

Discussion

Inhibition of neutrophil degranulation by MTC. The present work describes the inhibitory effect of the colchicine analogue MTC on enzyme release and microtubule polymerization in human neutrophils. In a comparative study, MTC was more effective than colchicine in inhibiting cell degranulation. We have found that high concentrations of colchicine were needed to inhibit enzyme release when preincubated for short periods of time with cells, and its microbule-inactive analogue lumicolchicine affected the enzyme release in human neutrophils to a similar extent. These results indicate that the early colchicine effect on degranulation of isolated human neutrophils is not microtubule specific. Only after prolonged preincubation times (30 or 60 min) was a significantly higher inhibitory effect of colchicine than lumicolchicine on cell degranulation observed. This would be consistent with the known rapid human cell penetration but slow binding to tubulin of colchicine that was shown by Taylor (39) in a classical study in cultured human K.B. cells. In contrast, MTC behaved as a very rapid and efficient compound in inhibiting granule release and in disrupting microtubules, after only a 5-min incubation with human neutrophils. The inhibitory capacity of MTC was found to be similar to that of nocodazole and taxol. The similar inhibitory capacity of different drugs affecting distinct aspects of the assembly/disassembly of microtubules supports the notion of the direct effect of MTC on microtubule disruption in living cells. Analogues of the two tubulin-binding portions of the MTC molecule are able to inhibit neutrophil degranulation, albeit at much higher concentrations than MTC. This could be explained by the presence of only one complementary portion in each analogue molecule. This result suggests that both parts of the MTC molecule and occupation of both binding subsites in the tubulin molecule (16) are required for effective microtubule disruption and enzyme release inhibition at low drug concentrations.

Unlike colchicine, the action of MTC on enzyme release inhibition and microtubule disruption was completely reversible. Recent evidence indicates that MTC is an effective and reversible inhibitor of microtubules and cell growth in PtK2 cultured cells (40). It is noteworthy that microtubule assembly inhibition of MTC *in vitro* is substoichiometric (18). This could explain the low MTC concentration required to inhibit enzyme release and cell growth.

Microtubules and neutrophil degranulation. The immunofluorescence technique let us study the MTC action on the microtubule network of neutrophils. Neutrophils adhered to glass exhibited approximately 30 microtubules/cell, originating from a single MTOC. However, we found striking alterations of microtubule assembly as a result of drug treatment and correlated these changes with drug inhibition of granule enzyme release. These effects on the microtubule network correlated well with the inhibitory action of MTC on enzyme release. However, there is an apparent lack of correlation between the strong effect of colchicine on microtubules and its weak effect on degranulation. This has to be approached with caution, because it involves the comparison of quantitative enzyme release measurements with essentially qualitative immunofluorescence observations, which were performed under conditions as close as technically possible, but not identical, to the release assays. Two hypothesis can be inferred from the results herein reported: (a) an intact microtubule network is not needed for degranulation, because colchicine is able to disrupt microtubules without an appreciable effect on degranulation and (b) degranulation is mediated by cytoplasmic microtubules. We favor this latter option because (a) the effects of MTC and MTPC on both the microtubule network and degranulation are distinct; (b) microtubule depolymerization and enzyme release inhibition induced by MTC are well correlated; (c) different drugs acting on microtubules are able to inhibit degranulation. In contrast, the effects of colchicine and lumicolchicine on degranulation at short preincubation times are not microtubule

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specific. A possible explanation for the apparently contradictory results obtained with colchicine at longer preincubation times on both the microtubule network and degranulation could be as follows: in 10^{-5} M colchicine-treated cells (30-min preincubation), secretion remains functional with the colchicine-resistant microtubules present under the experimental conditions used. Colchicine-induced microtubule depolymerization may be less extensive than that induced by MTC, due to the slow binding of colchicine to microtubules.

Disruption of microtubules by colchicine has been shown to produce an increase in the levels of cAMP (41, 42), as well as an inhibition of leukotriene B_4 formation (43) in neutrophils. Furthermore, the colchicine effects may reflect the capacity of colchicine to interact with various membrane proteins (44). The apparently contradictory results obtained with colchicine in relation to its capacity for disrupting microtubules and inhibiting enzyme release are in agreement with the recent observation of the binding of some colchicine analogues to tubulin without significant antiinflammatory activity (45). These observations suggest that the antiinflammatory action of colchicinoids may not be mediated totally through the microtubule system. Thus, caution must be taken when studying microtubule-related cellular functions with colchicine and certain colchicine analogues. The results herein reported suggest that, in addition to the known involvement of microtubules directing cytoplasmic granule movements, certain mechanisms that bypass the intact microtubule requirement could be induced during neutrophil activation. In this context, Carpen (46) has reported that natural killer cells are capable of lysing their targets by direct secretion of toxic materials, in the apparent absence of microtubules.

The results herein reported indicate that the loss of the middle ring in the colchicine molecule (Fig. 1) maintains a specific interaction with tubulin and avoids the slow and poorly reversible action of colchicine. In this regard, and owing to the low MTC concentration required to inhibit microtubule assembly, this drug could be considered an excellent tool to study microtubule-dependent cellular functions. However, we cannot presently rule out the possibility that MTC, by itself or by a microtubule-mediated process, might affect signal-transducing processes or might alter the level of intracellular second messengers, which could then in a secondary way inhibit the cellular responses.

Colchicine administered to humans has been shown to be effective in the treatment of several diseases, such as gout, but the doses required for these treatments have been reported to be toxic in several cases (47–49). In this regard, a potential therapeutic use might be investigated for MTC, due to its rapid, effective, and reversible action on microtubules and neutrophil degranulation. Secretion of neutrophil granule constituents mediates tissue damage in the inflammatory reaction. Therefore, inhibition of neutrophil degranulation by MTC could be of clinical interest in inflammation.

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