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Secretion of macrophage neutral proteinase is enhanced by colchicine

(elastase/collagenase/lysozyme/cytochalasin B)

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ABSTRACT **We have studied the effect of colchicine and related compounds on secretion of enzymes by thioglycollate-elicited mouse peritoneal macrophages in culture. Colchicine stimulated secretion of inducible neutral proteinase activities of elastase (EC 3.4.21.11), collagenase (EC 3.4.24.3), gelatinase (pepsin B; EC 3.4.23.2), and azocaseinase 2- to 6-fold for a period of several days, but inhibited the production and release of lysozyme (mucopeptide *N*-acetylmuramoylhydrolase; EC 3.2.1.17), a noninducible macrophage secretory product. Parallel changes were observed in cell morphology and secretion after treatment with colchicine, Colcemid, and vinblastine, but not with lumicolchicine, and these effects could be gradually reversed by withdrawal of colchicine. Cytochalasin B also stimulated secretion of elastase 2- to 3-fold, but did not influence release of lysozyme.**

These results demonstrate that tubulin-binding drugs may have opposite effects in macrophages than those usually reported for other experimental systems and also provide evidence for the nonparallel discharge of different macrophage secretion products.

Macrophages are specialized phagocytic cells that are widely distributed in the body and that play a central role in host defense against infection (1). Recent studies have shown that the mononuclear phagocyte can also display considerable secretory activity, since cultivated mouse macrophages are able to synthesize and release a variety of enzymes into their extracellular environment. These products include lysozyme (2), which is secreted continuously, independent of macrophage stimulation or phagocytic activity, and several inducible neutral proteinase activities, including elastase (3), collagenase (4), plasminogen activators (5, 6), and other less characterized proteinase¶ activities, which are produced and released in response to intraperitoneal stimulation by thioglycollate broth or endotoxin and after phagocytosis.

Cultivated mouse peritoneal macrophages provide a useful experimental system to investigate the sites of synthesis and storage and the intracellular pathway of these secretion products. Since colchicine and cytochalasin B have proved of interest in studying the role of microtubules, microfilaments, and the plasma membrane in secretion by a variety of cells (7), we have also studied the effects of these agents on secretion of macrophage enzymes. We report here that colchicine selectively stimulates secretion of elastase and some other neutral proteinase activities over prolonged periods of time, while inhibiting production and release of lysozyme. Cytochalasin B also stimulates secretion of elastase, but, in contrast, does not affect the release of lysozyme. The effects of colchicine on enzyme secretion occur in close parallel with morphologic evidence of cytoplasmic disorganization.

MATERIALS AND METHODS

Cell culture. Thioglycollate-stimulated, mouse peritoneal macrophages were harvested and cultivated in Dulbecco's medium with 15% acid-treated fetal bovine serum for 48 hr, as described (3). Macrophage conditioned medium was collected in the absence of serum from cells cultivated a further 24–96 hr in Dulbecco's medium supplemented with 0.2% lactalbumin hydrolysate or in Neuman-Tytell medium (3). Cell lysates were prepared in 0.2% wt/vol Triton X-100 (3).

Inhibitors. Colchicine (Sigma Chemical Co., Ltd., Kingston-upon-Thames, Surrey, U.K.) was dissolved in water (1 mg/ml) and kept dark at 4° or −70°. Lumicolchicine, a mixture of colchicine photoisomers, was prepared by ultraviolet irradiation of an ethanol solution of colchicine and assayed by its absorption spectrum (8, 9). Stock solutions of Colcemid (Ciba Lab., Horsham, Sussex, U.K.) and vinblastine sulfate (Lilly and Co., Ltd., Basingstoke, U.K.) were prepared in water and kept frozen. Cytochalasin B (Ralph Emmanuel, Wembley, Middlesex, U.K.) was dissolved in dimethyl sulfoxide (Me_2SO) (250 μg/ml, final Me_2SO concentration 10% vol/vol) and kept at 4°. All reagents were diluted in serum-free medium to the desired concentration and filter-sterilized before use. Macrophages that had been cultivated for 48 hr were washed three times and then incubated in the presence of inhibitors up to 96 hr, as indicated. Me_2SO and other appropriate controls were included at all times. Cell morphology was studied by phase contrast microscopy.

Enzyme Assays were performed on macrophage conditioned medium or on samples concentrated 10-fold by lyophilization, after dialysis against buffer, 10 mM Tris · HCl, 2 mM $CaCl_2$, pH 7.4 (4). Elastase (EC 3.4.21.11) was measured from zones of lysis in elastin-sodium dodecyl sulfate-agarose gels (3) using unconcentrated conditioned medium. Collagenase (EC 3.4.24.3) activity was usually measured in concentrated conditioned medium using reconstituted [^{14}C]glycine-labeled rabbit skin collagen (4); 1 unit of enzyme hydrolyzed 1 μg of collagen per min at 37°. Gelatinase (pepsin B; EC 3.4.23.2) activity was measured as described by Werb and Burleigh (10), using [^{14}C]glycine-labeled gelatin prepared by treating rabbit skin collagen for 30 min at 50°; 1 unit of enzyme hydrolyzed 1 μg of gelatin per min, at 37°, to peptides smaller than a molecular weight of 5000. Azocaseinase activity in concentrated conditioned medium was measured at pH 7.5, as described (3). Lysozyme (mucopeptide *N*-acetylmuramoylhydrolase; EC 3.2.1.17) activity

Abbreviation: Me_2SO, dimethylsulfoxide.

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¶ Z. Werb and S. Gordon, manuscript in preparation.

was determined from the initial rate of lysis of *Micrococcus lysodeikticus* (2). Samples were diluted in Dulbecco's medium containing 10% fetal bovine serum, to inhibit proteinases in macrophage conditioned medium which could enhance bacterial lysis. Results are expressed in terms of a rat lysozyme standard. Cathepsin D was assayed using [^{3}H]acetyl-labeled denatured ox hemoglobin as substrate (4). Cell protein was measured using egg lysozyme as standard (3).

Control experiments showed that the compounds and solvents used in these studies had no direct effect on enzyme activity. All assays were done in duplicate. Results are shown as the average and range of duplicate cultures, unless noted otherwise.

RESULTS

Effect of colchicine on macrophages

Morphology. Bhisey and Freed have described the striking alterations in macrophage motility and intracellular architecture which accompany depolymerization of microtubules by agents such as colchicine and vinblastine (11). Similar changes were noted in the present study. The thioglycollate-stimulated macrophages lost their characteristic bipolar appearance, acquired bizarre forms, and displayed spiky surface projections and blunt pseudopodia, a disrupted centrosphere region, and random mixing of intracellular organelles. The rate and extent of the morphologic changes were dose-related, with a uniform, maximal effect observed at a colchicine concentration of 1×10^{-6} M, or greater, and with no effect seen below 3×10^{-8} M. Macrophages exposed to colchicine for prolonged periods became rounded, but 80% of the cells remained adherent for as long as 3 days. The morphologic effects of colchicine treatment could be gradually reversed over a period of 1–2 days by repeated washing and cultivation in drug-free medium.

Elastase Secretion. The effect of colchicine treatment on elastase secretion by macrophages is illustrated in Fig. 1. Elastase levels in conditioned medium were stimulated 3-fold and continued to accumulate extracellularly at a constant rate for 3 days. Similar results have been reproduced in six independent experiments in which colchicine, at a concentration of 1 to 5×10^{-6} M, has stimulated elastase secretion between 3- and 6-fold. Fig. 2 shows a dose response experiment in which macrophage elastase activity increased progressively in the concentration range 3×10^{-8} M to 1×10^{-6} M. The decline seen here at 10^{-5} M was not observed in other experiments in which a constant level of stimulation was obtained between 1×10^{-6} M and 1×10^{-4} M. It is evident that colchicine also stimulated intracellular levels of elastase, but this represented less than 10% of extracellular activity at all concentrations tested. The stimulation of macrophage elastase was closely correlated with changes in cellular morphology.

Control mixing experiments between cell lysates and conditioned media from treated and untreated cultures revealed no stimulatory or inhibitory factors in these fractions. We therefore concluded that colchicine stimulated total elastase activity in macrophage cultures.

Other Enzymes. Since elastase is but one of several enzyme activities secreted by thioglycollate-stimulated macrophages (2, 4, 5), the effect of colchicine on some of these products was also investigated. Fig. 3 shows an experiment in which macrophage cultures were maintained, without medium change, in the presence or absence of colchicine for 4 days and sampled at intervals to determine enzyme secretion. This experiment showed that colchicine stimulated secretion of other neutral proteinase activities, such as collagenase and gelatinase, in parallel with elastase, but the lysozyme secretion was, on the other hand, inhibited. Kinetic analysis revealed that enhanced levels of proteinase activity could be detected within 6–12 hr of treatment and were maintained as long as 3–4 days, although some enzyme instability, e.g., collagenase, was noted after prolonged incubation. In striking contrast, the secretion of lysozyme was unaffected for at least 12 hr, but was then inhibited progressively.

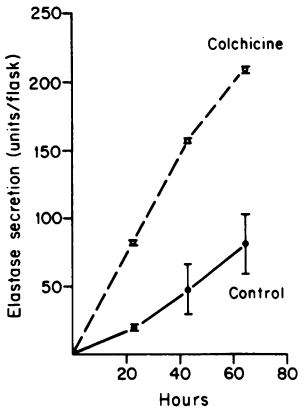

FIG. 1. Effect of colchicine on secretion of elastase by macrophages. Thioglycollate-stimulated macrophages (6×10^6) were cultivated in Dulbecco's medium + 15% acid-treated fetal bovine serum for 48 hr, washed, and then placed in Dulbecco's medium + 0.2% lactalbumin hydrolysate with or without 1.25×10^{-6} M colchicine. Conditioned medium was collected and replaced with fresh medium of similar composition at daily intervals. Result shows cumulative secretion by duplicate cultures.

Further evidence for a selective effect of colchicine on the activity and release of macrophage enzymes is given in Table 1. Whereas elastase, collagenase, and azocaseinase activities were stimulated 5-, 2-, and 5-fold, respectively, lysozyme levels were reduced by half, with depression of both intra- and extracellular enzyme, as noted before (2). Total levels of cathepsin D, a product which is predominantly retained within macrophages, were unaffected by colchicine and showed only a small increase in the proportion of extracellular enzyme, from 21 to 29%.

The inhibition of lysozyme production and secretion by colchicine showed a lag period of 12–36 hr in different ex-

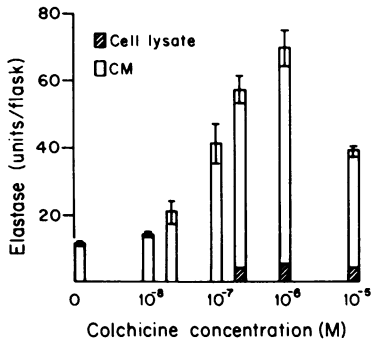

FIG. 2. Dose response of colchicine stimulation of macrophage elastase. Thioglycollate-stimulated macrophages (4×10^6) were treated with drug for 26 hr. There was no significant difference between the cell protein recovered in control (0.39 ± 0.007) and colchicine-treated cultures (0.36 ± 0.042, mean ±SD). CM, conditioned medium.

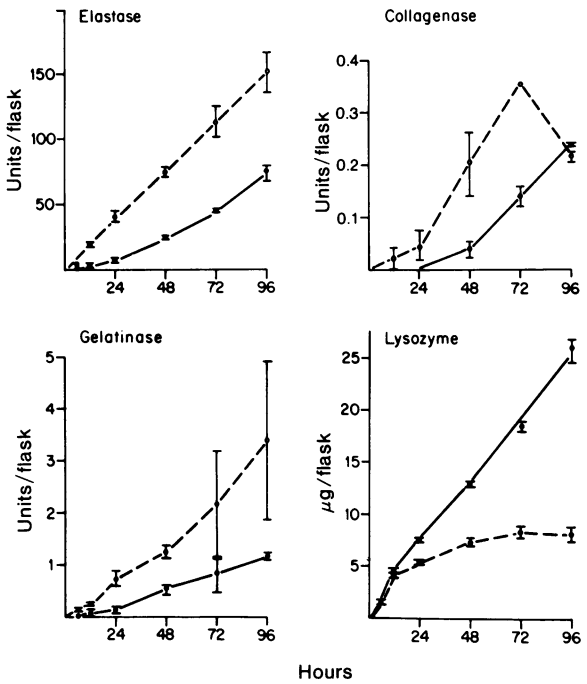

FIG. 3. Effect of colchicine on secretion of macrophage enzymes. Thioglycollate-stimulated macrophages (6×10^6) were cultivated in Dulbecco's medium + 15% acid-treated fetal bovine serum for 48 hr, washed, and then placed in Neuman-Tytell medium with or without 5×10^{-6} M colchicine. Portions of conditioned medium were removed at times shown and replaced with fresh medium. Average values of duplicate cultures were corrected for portion removed. (O- - -O) Colchicine-treated; (●—●) control.

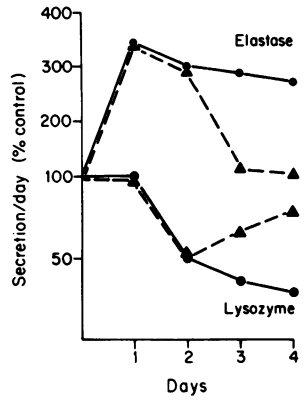

FIG. 4. Reversibility of colchicine effect on secretion of macrophage elastase and lysozyme. Thioglycollate-stimulated macrophages (6×10^6) were cultivated for 48 hr in Dulbecco's medium + 15% acid-treated fetal bovine serum, washed, and placed in Dulbecco's medium + 0.2% lactalbumin hydrolysate with or without colchicine (1×10^{-6} M) for 1 or 4 days. Conditioned medium was collected and replaced with fresh medium daily. (●—●) 4 days of medium with drug; (▲- - -▲) 1 day treatment with drug followed by 3 days in drug-free medium. Each point shows average of duplicate cultures.

periments, but was invariably complete by 48 hr. Control mixing experiments showed that no inhibitor of lysozyme was present in medium conditioned by colchicine-treated macrophages.

Reversibility. The morphologic effects of colchicine could be gradually reversed within 1–2 days of drug washout. As shown in Fig. 4, stimulation of elastase and inhibition of lysozyme secretion were reversed in parallel with the restoration of normal morphology, after a lag period of 1 day.

Effect of related compounds

When Colcemid and vinblastine, compounds which also depolymerize microtubules, were tested at a concentration of 2×10^{-6} M, they were as effective as colchicine in producing the characteristic morphologic effects, stimulating elastase and collagenase and inhibiting lysozyme secretion. In contrast, lumicolchicine, which lacks the action of colchicine on microtubules but retains some of its activity against plasma membrane transport sites (9), failed to influence either cell morphology or elastase secretion, even at 1000-fold greater concentrations, as shown in Table 2. Similarly, 1×10^{-6} M lumicolchicine failed to inhibit secretion of lysozyme over a period of 3 days. These experiments confirmed that the modification of enzyme secretion by colchicine and related compounds was closely correlated with their effectiveness against microtubules.

Effect of cytochalasin B

Axline and Reaven (12) have described effects of cytochalasin B on cultivated mouse macrophages, including characteristic changes in cell shape, cessation of plasma membrane movement, and disorganization of certain subplasmalemmal microfilament bundles. In the present studies thioglycollate-stimulated macrophages exposed to cytochalasin B (1–10 μg/ml) showed similar thickening of the centrosphere region and a thin arborized peripheral cytoplasm. The cells remained adherent and apparently unchanged during 4 days of continuous treatment. Treated cultures became more alkaline, presumably because of inhibition of hexose transport (12). These effects were rapidly reversed by removing the drug.

Fig. 5 shows an experiment in which exposure to cytocha-

Table 1. Effect of colchicine on intra- and extracellular activity of macrophage enzymes*

	Elastase, units	Collagenase,† milliunits	Azocaseinase,† milliunits	Lysozyme, μg	Cathepsin D, milliunits
Control					
Conditioned medium	2.8 ± 0.91	36 ± 1.4	59 ± 9.9	40 ± 0.71	31 ± 6.4
Cell lysate	0.2 ± 0.28	0	ND	4.1 ± 0.071	114 ± 20
Colchicine					
Conditioned medium	14.0 ± 2.9	70 ± 0.71	295 ± 7.1	19 ± 0.71	46 ± 0
Cell lysate	1.2 ± 1.1	0	ND	1.5 ± 0.071	110 ± 0.71

ND, not done.

* Thioglycollate-stimulated macrophages were cultivated in Dulbecco's medium + 15% acid-treated fetal bovine serum for 48 hr, then washed and exposed to 4×10^{-6} M colchicine in serum-free medium for 24 hr. Enzyme activity is expressed per mg of cell protein per 24 hr and is shown as mean ± SD. Results of two experiments with duplicate cultures.

† Dialyzed and concentrated before assay.

Table 2. Effect of colchicine and lumicolchicine on elastase secretion*

Treatment	Elastase secretion	
	Units/flask	% Control
None	22	100
Ethanol, 2%	22	100
Colchicine		
10^{-5} M	85	390
10^{-6} M	65	300
10^{-7} M	80	360
Lumicolchicine		
10^{-4} M†	15	68
10^{-5} M	23	110
10^{-6} M	23	110
10^{-7} M	22	100

* Thioglycollate-stimulated macrophages (7×10^6) were cultivated for 48 hr in Dulbecco's medium + 15% acid-treated fetal bovine serum, then washed and treated with drugs in Dulbecco's medium + 0.2% lactalbumin hydrolysate for 26 hr. Cell protein at end of experiment: control, 0.54 mg; colchicine (10^{-6} M), 0.46 mg; lumicolchicine (10^{-6} M), 0.46 mg. Cell lysate elastase: control, 1 unit; colchicine, 5 units; lumicolchicine, 0.5 unit.

† 2% ethanol, final concentration.

lasin B stimulated the release of macrophage elastase 2- to 3-fold over 3 days, but failed to influence the release of lysozyme, as noted previously (2). In three independent experiments the level of stimulation of elastase activity by cytochalasin B varied between 140 and 300%. Control cultures treated with Me_2SO showed no effect on macrophage morphology or secretion of elastase. Stimulation of elastase secretion by cytochalasin B was dose-dependent, with a maximal effect at 3–4 μg/ml and none observed at concentrations lower than 1 μg/ml. Other experiments showed the levels of intracellular elastase were also increased, but still represented less than 5% of total enzyme activity per culture. The effect on elastase secretion was rapidly reversed by washing out the drug. These studies indicated that cytochalasin B could also stimulate the selective accumulation and secretion of elastase activity by thioglycollate-elicited macrophages.

DISCUSSION

These results demonstrate that different macrophage products are discharged independently and that their secretion can be either stimulated or inhibited by tubulin-binding drugs. This conclusion contrasts with the general observation that colchicine inhibits secretion in a wide variety of systems (7), although previous reports have shown that colchicine can also stimulate secretion of collagenase by explants of synovial tissue (13) and of steroids by adrenal tumor cells in culture (14). The present studies, of course, do not indicate whether differential secretion and response to drugs can be attributed to the same macrophages.

The mechanisms that could account for enhanced levels of neutral proteinase activity in macrophage cultures include activation of enzymes and increased synthesis, decreased degradation, and diminished production of inhibitors (15). Since several distinct proteinase activities are elevated, such inhibitors would be expected to show a rather broad specificity. Colchicine has little effect for several hours on total protein synthesis in the macrophage (16), but the species formed could be substantially modified in the presence of colchicine (13). The continued accumulation of

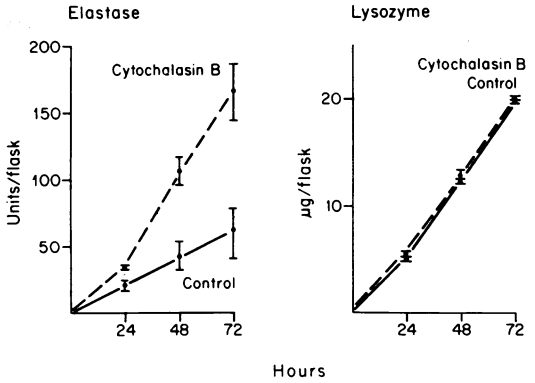

FIG. 5. Effect of cytochalasin B on secretion of macrophage enzymes. Thioglycollate-stimulated macrophages (6×10^6) were cultivated in Dulbecco's medium + 15% acid-treated fetal bovine serum for 48 hr, washed, and placed in Dulbecco's medium + 0.2% lactalbumin hydrolysate + 0.16% Me_2SO with or without 4 μg/ml of cytochalasin B. Conditioned medium was collected and replaced with identical fresh medium at daily intervals. Cumulative secretion by duplicate cultures is shown. (●- - -●) Cytochalasin B treated; (●—●) controls.

enzyme activity after treatment and its prevention by cycloheximide‖ provide some evidence for increased synthesis, but further studies are needed to rule out alternate explanations.

Although a direct effect on microtubules has not been demonstrated in these studies, morphologic evidence suggests that modification of enzyme secretion by colchicine and related agents occurs in parallel with depolymerization of macrophage microtubules (11, 16). The efficacy of low doses of colchicine and inactivity of lumicolchicine are also consistent with such a relationship (9). Antimitotic effects can be excluded in the present studies, although a proportion of thioglycollate-stimulated macrophages may express a potential to proliferate under different culture conditions (17). The role of microtubules in modifying macrophage secretion remains unclear, however, and additional cellular targets should also be kept in mind. Colchicine is known to alter the function (9, 16) and topographic distribution (18) of plasma membrane transport sites, to inhibit some assays of pinocytic activity (11, 16), and to prevent induction of lysosomal acid hydrolases by endocytosis in macrophages (16). Abnormal function of the Golgi apparatus (19, 20) and stimulation of cellular autophagy (21) have also been reported for other cells.

Similar questions can be raised about the mechanism of action of cytochalasin B, although a different cellular target is probably involved. Cytochalasin B is known to disrupt macrophage microfilaments and to inhibit hexose transport across the plasma membrane (12). Cytochalasin treatment also causes enhanced release of collagenase from fibroblasts (22) and promotes the release of lysosomal acid hydrolases from macrophages (23), but the latter effect is due to redistribution of intracellular enzyme, rather than increased total activity, as noted here.

Although the action of colchicine and cytochalasin B on production and the intracellular pathway of lysozyme and proteinases remains obscure, these drugs enable us to differentiate among different macrophage products and their regulation. It is uncertain if these drugs stimulate all inducible neutral proteinase activities in parallel. Preliminary experiments have shown that plasminogen activator levels decline

‖ S. Gordon and Z. Werb, unpublished observations.

after colchicine treatment**, and the effect of cytochalasin B on other enzyme activities is not known. Previous studies showed that secretion of the proteinases is regulated by two distinct processes, namely, macrophage activation and uptake and storage of particulate materials (6, 3, 4). The effect of these drugs on macrophage secretion is independent of thioglycollate stimulation since unstimulated cells respond similarly and is distinct from phagocytosis since enzyme secretion by thioglycollate-elicited macrophages cannot be enhanced by ingestion and storage of latex‖ (6). The use of colchicine and cytochalasin B has, therefore, made it possible to distinguish additional mechanisms for regulation of macrophage secretory activity.

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** S. Gordon, unpublished observations.