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STIMULATION OF THE SECRETION OF PLASMINOGEN ACTIVATOR FROM ACTIVATED MURINE MACROPHAGES BY MICROTUBULE DISRUPTING AGENTS AND DEUTERIUM OXIDE

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Abstract—Murine peritoneal macrophages activated *in vivo* with thioglycollate broth secrete plasminogen activator and other neutral proteinases. The secretion of plasminogen activator by these cells is potentiated by continuous culture with the microtubule destabilizing drugs colchicine, demecolchicine, vinblastine and nocodazole but not by the colchicine analogues trimethylcolchicinic acid and colchicoside which do not destabilize microtubules. Pulse treatment with colchicine for 2 hr or with nocodazole for 16 hr also stimulated plasminogen activator secretion. Deuterium oxide, which stabilizes microtubules, also stimulated secretion of plasminogen activator and failed to antagonize the effects of destabilizing agents on enzyme secretion. It is concluded that secretion of plasminogen activator is not dependent on an intact microtubular system.

Macrophages secrete several neutral proteolytic enzymes which are not derived from the lysosomal system. Collagenase, elastase, plasminogen activator (PA)* and several other incompletely characterized proteinases have been detected in the media from cultures of mouse and guinea pig macrophages [1–4]. Secretion of these enzymes by activated macrophages may explain the destruction of connective tissue which occurs in chronic inflammatory lesions where the macrophage is considered to play an important role [5]. In comparison to the quantities found in the culture medium, intracellular levels of these enzymes are low, suggesting that they are not stored in significant quantities but are secreted soon after synthesis [3]. Macrophages stimulated *in vivo* with thioglycollate broth or bacterial endotoxin secrete appreciable quantities of these enzymes, although endotoxin stimulated cells require a further phagocytic stimulus *in vitro* for optimal secretion [6].

The integrity of intracellular microtubules is essential for a variety of secretory processes. Microtubule-disrupting agents such as colchicine and the *Vinca* alkaloids inhibit the secretion of plasma proteins from hepatocytes [7], insulin from beta cells [8], procollagen from embryo chick tendon and cartilage cells [9], growth hormone and prolactin from pituitary slices [10] and the release of catecholamines from the adrenal medulla [11] and histamine from mast cells [12] and basophils [13]. In the macrophage colchicine inhibits both phagocytosis [14] and the release of lysosomal enzymes [15] and has been reported to inhibit the secretion of plasminogen activator [16]. However, the secretion of other neutral proteinases and of pyrogen by the macrophage is stimulated by colchicine [17, 18].

We have studied the release of plasminogen activator (PA) from mouse macrophages stimulated *in vivo* with thioglycollate broth by measuring their ability to degrade ^{125}I-labelled fibrin *in situ*. We have found that colchicine stimulates the secretion of PA from these cells and that culture in deuterium oxide (D_2O) augments rather than inhibits this stimulation.

MATERIALS AND METHODS

Materials. Male albino mice (CFLP) weighing 25–40 g were supplied by our own breeding unit or by Anglia Laboratories, Huntingdon Research Centre, Huntingdon, U.K.

Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) were obtained from Gibco Bio-cult, Paisley, Scotland. Thioglycollate medium was obtained from Difco Laboratories, MI, U.S.A., and bovine fibrinogen (Type I), thrombin, trypsin (type III—bovine pancreas), colchicine, demecolchicine and vinblastine from Sigma, London, U.K. Trimethylcolchicinic acid and colchicoside were purchased from K & K Laboratories, New York, U.S.A., and nocoadazole from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Deuterium oxide was from Fluorochem, Glossop, U.K. and ^{125}I-labelled human fibrinogen was supplied by the Radiochemical Centre, Amersham, U.K.

Culture medium. DMEM was reconstituted from powder form with distilled water and supplemented with 2.3 g/l. $NaHCO_3$, 5.67 g/l. HEPES (*H*-2-hydroxyethylpiperazine-*N*-2-ethane sulphonic acid), 100 mg/l. penicillin and streptomycin and 10% acid

* Abbreviations used: PA, plasminogen activator; DMEM, Dulbecco's Modified Eagles Medium; FBS; foetal bovine serum; ATFBS, acid treated foetal bovine serum; D_2O, deuterium oxide; TMCA, trimethyl colchicinic acid; cyclic AMP, cyclic adenosine-3′5′-monophosphate.

treated foetal bovine serum (ATFBS). The serum was treated with acid to remove inhibitors of plasmin [3]. DMEM-AFTBS was sterilized by millipore (0.22 μm) filtration before use.

Collection of macrophages. Mice were injected intraperitoneally with 1 ml of thioglycollate broth and four days later were killed by asphyxiation in carbon dioxide. The peritoneal cavities were washed out with 5 ml DMEM-AFTBS containing 10 I.U./ml heparin (Pularin, Evans Medical, Speke, U.K.) and the pooled washings adjusted to 1 to 2×10^6 white cells per ml before applying to petri dishes.

Preparation of [^{125}I]fibrin coated petri dishes. These were prepared essentially as described by other workers [19]. Purified bovine fibrinogen [20] was diluted to a concentration of 2.2 mg/ml in phosphate-buffered saline and ^{125}I-labelled human fibrinogen added to give 10^6 dpm/ml. After sterilization by filtration 0.1 ml aliquots were spread on 5 cm petri dishes (Nunc, N-1400) and the dishes dried for 2 days at 37° in a dry incubator. The fibrinogen was converted to fibrin by overnight incubation with 5 ml DMEM containing 10% FBS. The dishes were washed twice with sterile Tris-phosphate buffer (0.24 M Tris–HCl, 0.14 M NaCl, 0.05 M KCl, 0.0037 M Na_2HPO_4, pH 7.4) and stored at −25° until required. For use the dishes were allowed to reach room temperature and washed once with sterile Tris-phosphate buffer.

Measurement of plasminogen activator secretion. Aliquots of the pooled peritoneal washings containing 5×10^6 cells were pipetted onto the [^{125}I]fibrin coated dishes, cultured at 37° for 3 hr in 95% air : 5% CO_2 and non-adherent cells removed by washing three times with phosphate-buffered saline. The resulting monolayer was cultured for a further 24 hr in 5 ml fresh DMEM-ATFBS before any experimental treatment. For each experiment the medium was replaced every 24 hr and PA secretion estimated by measuring the release of ^{125}I into the culture medium using a Nuclear Enterprises 8132 counter. ^{125}I release was corrected for release from dishes incubated with medium but without cells (approximately 1 per cent of the total each day). Residual radioactivity remaining on the dishes at the end of the experiment was removed by incubation overnight with 0.1 mg/ml trypsin in phosphate-buffered saline and the release of ^{125}I from each dish was expressed as a percentage of the total radioactivity. All treatments were carried out in triplicate.

Drug treatment. Colchicine, demecolchicine, trimethylcolchicinic acid, colchicoside and vinblastine were all dissolved in DMEM to give stock solutions of 10^{-3} M. Nocodazole stock solutions contained 1% ethanol and 0.04 M sodium hydroxide in DMEM. All dilutions were made with DMEM-ATFBS and drug-free controls contained the necessary vehicle. Drug solutions or vehicle controls were added to the cells after the first 24 hr culture period and removed, counted and renewed at 24-hr intervals for a further 3 days.

Macrophages were pulse-treated with drugs by incubating the cells in medium containing drug for 2 hr on the second day of culture. The cells were then washed three times with sterile phosphate-buffered saline before fresh control medium was added for measurement of fibrinolysis. In preliminary experiments the amount of fibrin solubilized during the pulse period was found to be small (<0.4 per cent of total radioactivity) and so it was not included routinely in measurements of PA secretion. However, in experiments where the pulse period was extended beyond 2 hr the pulse medium was retained for ^{125}I measurement.

Culture medium supplemented with deuterium oxide (D_2O) was made by mixing DMEM made with distilled water with DMEM made with 98% D_2O to give the appropriate concentration of D_2O. Continuous and pulse treatments with D_2O supplemented media were carried out as described above.

RESULTS

In preliminary experiments fibrinolysis by TGS macrophages was tested for dependence upon the presence of plasminogen. Plasminogen was removed from ATFBS by passage through a column of lysine–sepharose 4B [21]. Fibrinolysis by macrophages cultured in DMEM supplemented with 10% plasminogen depleted ATFBS was barely detectable but could be restored to control values by the addition of purified plasminogen to the cultures. The release of ^{125}I from TGS macrophage monolayers was therefore due to the release of PA from these cells rather than to fibrinolytic proteases.

PA secretion by stimulated macrophages. Thioglycollate-stimulated (TGS) cells secreted variable quantities of PA in different experiments, an observation which has been reported previously and attributed to variations in the response to thioglycollate broth [16]. For this reason a direct comparison of ^{125}I release between different experiments was often impossible. To facilitate evaluation of drug effects the release of ^{125}I from drug-treated cultures was expressed as the ratio between release in the presence and absence of drug in the same experiment.

The effect of colchicine. Colchicine (10^{-5}–10^{-7} M) present in the culture medium for the whole of the 3-day experimental period doubled PA secretion. Below 10^{-7} M colchicine had no effect (Table 1). A 2-hr pulse of colchicine on the second day of culture also produced a stimulation of secretion (Table 1), although this was less marked and required higher concentrations than did continuous culture with the

Table 1. The effect of colchicine upon PA secretion by TGS macrophages*

Colchicine concentration (M)	Ratio of test: control accumulative ^{125}I release after 4 days culture ± S.E.M.	
	Continuous	Pulse
1×10^{-4}	—	1.87 ± 0.37
1×10^{-5}	2.21 ± 0.11	1.92 ± 0.22
1×10^{-6}	2.05 ± 0.05	1.31 ± 0.08
1×10^{-7}	1.92 ± 0.15	1.20 ± 0.20
1×10^{-8}	0.86 ± 0.07	1.07 ± 0.08
1×10^{-10}	0.84 ± 0.08	—

* Control ^{125}I release varied from 6.0 to 32.3 per cent in different experiments.

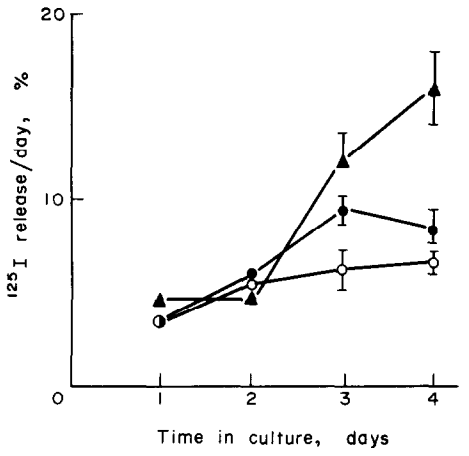

Fig. 1. The effect of colchicine on PA secretion from TGS macrophages. Cells were exposed to colchicine for the last three days of culture. Results are means ± S.D. of two experiments carried out in triplicate. ▲, TGS cells in medium + 10^{-6} M colchicine; ●, TGS cells given a 2-hr pulse of 10^{-6} M colchicine on day 2; ○, TGS cells in normal medium.

drug. With both continuous exposure and pulse treatment a 24-hr lag period was observed before stimulation of enzyme secretion occurred (Fig. 1). Macrophages treated with a pulse of colchicine showed maximal stimulation of PA secretion 48 hr after exposure to the drug (Fig. 1) showing that continuous exposure was necessary for colchicine to maintain its stimulatory effect on enzyme secretion.

Macrophages maintained in medium containing 10^{-5}–10^{-7} M colchicine showed the characteristic changes in shape that have been described by other workers [22]. Cells pulsed with colchicine, however, had regained their normal morphology 24 hr after removal of the drug and appeared normal at the end of the experiment.

Colchicine analogues and vinblastine. Demecholchicine (*N*-desacetyl-*N*-methylcolchicine) will disaggregate microtubules and inhibit phagocytosis *in vitro*, as does colchicine, but other close structural analogues trimethylcolchicinic acid (TMCA) and colchicoside (2-desmethylcolchicine glucoside) do not have these properties [23]. Vinblastine also interferes with the aggregation of microtubules but does not bind to the same site on tubulin as does colchicine [24]. These compounds were used to investigate whether stimulation of PA secretion by colchicine could be related to its ability to interfere with microtubule stability. Continuous exposure of TGS cells to 10^{-6} M demecolchicine or vinblastine caused stimulation of PA secretion, although both compounds were inactive when presented in a 2-hr pulse (Table 2). Colchicoside and TMCA did not stimulate PA secretion either when given continuously or as a pulse and the cells were unchanged morphologically by these treatments, whereas continuous exposure to vinblastine or demecolchicine induced morphological changes similar to those caused by colchicine.

Nocodazole. Nocodazole {methyl[5-(2-trienylcarbonyl)-L-H-benzimidazol-2-yl]carbamate} is a recently introduced synthetic microtubule destabilizer chemically unrelated to colchicine or the *Vinca* alkaloids but which produces identical cytological effects [25]. Nocodazole binds to the same site on the tubulin molecule as does colchicine but differs from colchicine in that the binding is rapidly reversible [26]. Nocodazole was used to confirm that microtubule destabilizers stimulate PA secretion and to investigate further the effect of a pulse of colchicine. Table 3, Column 1 shows that Nocodazole stimulates PA secretion when given continuously and so has an effect identical with that of other destabilizers. Nocodazole given as a 2-hr pulse did not stimulate PA secretion but exposure of TGS cells to nocodazole for increasing pulse times led to a

Table 2. The effect of colchicine analogues and vinblastine on PA secretion by TGS macrophages*

Compound	Concentration (M)	Ratio of test: control accumulative ^{125}I release after 4 days ± S.E.M.	
		Continuous	Pulse
Demecholchicine	1×10^{-4}	—	0.87 ± 0.08
	1×10^{-6}	1.53 ± 0.16	0.91 ± 0.08
	1×10^{-8}	0.75 ± 0.20	—
Trimethyl colchicinic acid	1×10^{-4}	—	0.72 ± 0.09
	1×10^{-6}	0.79 ± 0.18	0.86 ± 0.08
	1×10^{-8}	0.87 ± 0.21	—
Colchicoside	1×10^{-4}	—	0.97 ± 0.04
	1×10^{-6}	0.97 ± 0.12	0.83 ± 0.05
	1×10^{-8}	0.66 ± 0.05	—
Vinblastine	1×10^{-4}	—	†
	1×10^{-6}	1.84 ± 0.07	0.94 ± 0.09
	1×10^{-8}	0.91 ± 0.07	—

* Control ^{125}I release varied from 9.9 to 34.8 per cent in different experiments.
† Cytotoxic at this concentration.

Table 3. Comparison of the effects of colchicine and nocodazole on PA secretion by TGS macrophages maintained in normal culture medium and on PA secretion by macrophages maintained in culture medium supplemented with D_2O

Treatment		Accumulative % ^{125}I release after 4 days ± S.D.	
		Normal DMEM	DMEM + 32% D_2O
Control	Expt. I	21.66 ± 1.08	34.04 ± 3.04
	Expt. II	6.10 ± 0.58	26.34 ± 1.48
Colchicine	Expt. I	48.44 ± 3.63	57.91 ± 2.43
(1×10^{-5} M)	Expt. II	35.49 ± 9.75	75.14 ± 0.48
Nocodazole	Expt. I	50.69 ± 1.12	66.50 ± 1.94
(1×10^{-5} M)	Expt. II	20.23 ± 7.11	27.50 ± 3.50

progressive increase in stimulation of PA (Fig. 2) reaching a maximum after 16 hr exposure.

Interaction between microtubule inhibitors and deuterium oxide. Deuterium oxide D_2O is believed to stabilize microtubules [27] and to antagonize the effect of destabilizing agents [11–13]. However, Table 4 and the second column of Table 3 show that TGS cells cultured continuously in medium supplemented with D_2O secreted up to three times the amount of PA secreted by control cultures in normal medium. Macrophage cultures pulse-treated with D_2O-supplemented media were not stimulated to secrete PA above control levels (Table 4) even if the pulse time was extended to 24 hr. Medium containing D_2O had marked effects on cell morphology, producing approximately equal numbers of small rounded cells and large, highly spread cells with many cytoplasmic processes. Cell survival in medium containing more than 60 per cent D_2O was poor.

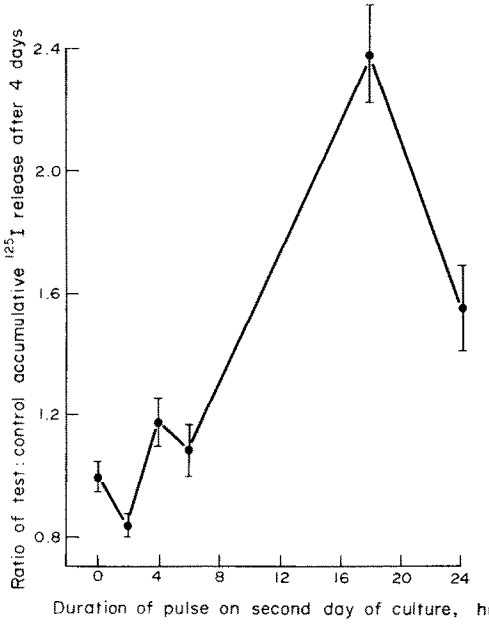

Fig. 2. The effect of pulse treatment with 10^{-5} M nocodazole on PA secretion from TGS macrophages. Cells were given 10^{-5} M nocodazole for increasing periods during the second day of culture. Medium was changed daily for four days and the effect of nocodazole expressed at the ratio of ^{125}I release in nocodazole-treated cells to control cells. Results are the mean ± S.D. of two experiments carried out in triplicate.

When colchicine or nocodazole was given in combination with medium containing 32 per cent D_2O PA secretion was enhanced above control cultures in 32 per cent D_2O alone (Table 3, Column 2). In addition, medium supplemented with D_2O did not antagonize the effect of colchicine or nocodazole on cell morphology.

DISCUSSION

The secretion of neutral proteinases by macrophages requires protein synthesis [1, 2] and there is no evidence of any significant intracellular accumulation of these enzymes following stimulation either *in vivo* [1, 2] or *in vitro* [5]. In all cases the effect of stimulation is to increase the quantity of enzyme which can be recovered from the culture medium. It appears, therefore, that macrophage neutral proteinases are secreted shortly after synthesis and that stimulation of enzyme secretion can be equated with stimulation of enzyme synthesis.

The central observation of this paper, that colchicine and other microtubule destabilizers stimulate PA secretion, contradicts the report of Vassalli *et al.* [16] but is in broad agreement with those of Gordon and Werb [17] who reported that colchicine stimulated the secretion of four neutral proteinases by macrophages. In general, it has been found that the secretion of PA from activated macrophages parallels the release of other neutral proteinases and it seems unlikely that colchicine would exert a differential effect on the secretion of one or more of these enzymes. The release of pyrogen, another product of activated macrophages that may share the same

Table 4. PA secretion by TGS macrophages maintained in culture medium supplemented with different concentrations of D_2O

Treatment	Ratio of test: control ^{125}I release after 4 days ± S.E.M.	
	Continuous*	Pulse†
16% D_2O	1.18 ± 0.07	—
32% D_2O	2.97 ± 0.67	1.06 ± 0.14
56% D_2O	2.75 ± 0.30	0.97 ± 0.12
80% D_2O	1.21 ± 0.10	—

* Control % ^{125}I release values = 6.1 and 19.5.
† Control % ^{125}I release values = 7.8 and 9.9.

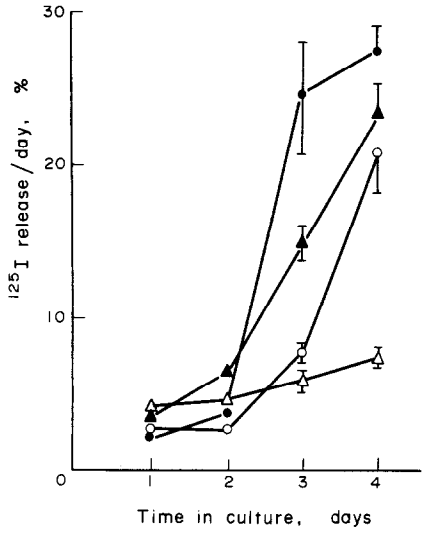

Fig. 3. The effect of 10^{-5} M colchicine on PA secretion by TGS macrophages maintained in normal medium and in medium supplemented with 32% deuterium oxide. Cells were exposed to colchicine and or D_2O for the last three days of culture. Medium was changed daily and ^{125}I release expressed as a percentage of the total ^{125}I on the dish. ●, TGS cells in medium containing 32% D_2O and 10^{-5} M colchicine; ○, TGS cells in medium containing 32% D_2O; ▲, TGS cells in normal medium containing 10^{-5} M colchicine; △, TGS cells in normal medium.

synthetic–secretory pathway as the neutral proteinases, has also been reported to be stimulated by colchicine [18]. Although colchicine inhibits secretion in a wide variety of systems there are instances where stimulation of secretion has been reported. Colchicine stimulates secretion of steroids from cultured adrenal cortex cells [28], lysosomal glycosidases from human fibroblasts [29], PA from a pig kidney cell line [30] and both collagenase and prostaglandins from rheumatoid synovial tissue [31, 32].

The results suggest that the ability of colchicine to stimulate PA secretion is related to the ability of the drug to destabilize microtubules. Thus colchicine analogues which destabilize microtubules also stimulate PA secretion. Vinblastine, a destabilizing agent structurally unrelated to colchicine and which binds to a different site on tubulin [24], also stimulates PA secretion, as does another structurally distinct destabilizer nocodazole. In contrast, the colchicine analogues TMCA and colchicoside neither destabilize microtubules nor stimulate PA secretion.

Colchicine, but not demecolchicine, vinblastine or nocodazole, stimulated PA secretion following pulse treatment of the macrophages for 2 hr. It is not known whether this effect is due to persistence of the drug within the cell or whether a transient destabilization of microtubules during the time of exposure is sufficient to initiate a series of events leading to increased synthesis and secretion of PA. Frankel [33] observed that, following a 2-hr exposure to 2×10^{-7} M colchicine, all the microtubules present in cultured mouse macrophages had depolymerized and that 1 hr after the removal of the drug the microtubules had reformed. However, in our experiments significant stimulation of PA secretion following a 2-hr pulse was only observed at concentration of 10^{-6} M or above. Although we have not carried out experiments to determine the state of assembly of microtubules directly under these conditions we observed that the cells did not regain their normal morphology until approximately 24 hr after withdrawal of colchicine, suggesting that at higher concentrations than that used by Frankel the effects of colchicine may be prolonged beyond the period of exposure to the drug. Pulse experiments with nocodazole indicate that prolonged exposure to this readily reversible drug is required to stimulate PA secretion. This observation and the persistance of morphological changes following pulse treatment with colchicine support the view that the microtubules must remain in the disaggregated state for several hours for PA secretion to be stimulated.

The mechanism by which microtubule destabilizing agents stimulate PA secretion is unknown. The effect is not exclusive to TGS cells as we have observed the same phenomenon in cells taken from unstimulated mice and from mice injected intraperitoneally with bacterial endotoxin (unpublished observation). The secretion of PA from macrophages stimulated *in vivo* with endotoxin is induced by the phagocytosis of indigestible particles such as latex or carbon microphefes [6] and there is evidence that the release of digestion products from phagolysosomes may play a role in regulating the synthesis of hydrolytic enzymes within the macrophage [34]. In this context the effect of the lectin Concanavalin A (Con A) provides an interesting analogy. Uptake of Con A into macrophage phagosomes causes vacuole formation and retardation of phagolysosome formation, as shown by electron micrographs and by a reduction in the degradation of internalized proteins [35]. Interference with the process of fusion between lysosomes and incompletely internalized phagosomes may be the method by which both colchicine [36] and Con A [15] inhibit the release of lysosomal enzymes from TGS cells. Con A, however, at concentrations which cause vacuolization, stimulates PA secretion from macrophages [37]. A reduction in the degradation of internalized proteins caused by the ability of colchicine to prevent phagolysosome formation may produce a situation parallel to that caused by the uptake of indigestible particles, thus stimulating the secretion of neutral proteinases. There is evidence that colchicine retards protein degradation in macrophages [38] and other cell types [29], although some workers have failed to confirm this [39].

Alternative mechanisms for the action of microtubule destabilizers on the secretion of PA may be related to their ability to disturb the distribution and mobility of surface receptors [40, 41] or to disrupt subcellular organisation causing synthetic or secretory abnormalities [7].

Deuterium oxide antagonizes the effects of colchicine in several systems, apparently by increasing the formation and stability of microtubules [11, 12, 13, 27]. In view of the close correlation between the destabilizing ability of the colchicine analogues, vinblastine and nocodazole, and stimulation of PA secretion, the failure of D_2O to antagonize the stimulatory effects of colchicine on PA

secretion and the ability of D_2O itself to promote secretion are difficult to interpret. Previous work has usually involved much shorter incubation times than those used in these experiments and it may be that in long term culture systems other than microtubules may be affected by D_2O. In seven out of eight different secretory systems reviewed by Gillespie [42] the effects of D_2O opposed those of cyclic AMP. This led her to suggest that D_2O was specifically antagonizing effects of intracellular cyclic AMP. Agents which raise the intracellular concentration of cyclic AMP inhibit PA secretion by activated macrophages [16] so D_2O may promote PA secretion by antagonizing intracellular cyclic AMP.

In conclusion, therefore, our observations demonstrate that in activated macrophages the secretion of plasminogen activator, unlike the majority of secretory processes but in common with the secretion of other neutral proteinases [17, 18], is not dependent upon an intact microtubular system.

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