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Influence of colchicine derivatives on lysosomal enzyme release from polymorphonuclear leukocytes and intracellular levels of cAMP after phagocytosis of monosodium urate crystals

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Colchicine and its analogues belong to the group of microtubular inhibitors [1]. Microtubules are involved in the transport towards lysosomes of extracellular material wrapped in vesicles. Drug-induced tubulostasis may lead to reduced digestion of phagocytozed material and could prevent metabolic transformation of this material. These events possibly play a role in the inhibition of the generation of inflammatory mediators [2]. Colchicine does not stabilize isolated lysosomes [3], so its primary action on polymorphonuclear functions (release of lysosomal enzymes and chemotactic factors, motility, etc.) is possibly mediated through its antimicrotubular activity [4].

Some of the colchicine derivatives exhibit similar effects as the parent compound, e.g. demecolcine and deacetylcolchiceine, and are equivalent to colchicine in anti-gout activity [5]. Deacetylcolchiceine is nearly as effective as colchicine in inhibition of urate-stimulated motility of PMN [6] and less active in urate-induced paw swelling in rat [7].

Since enzyme release from PMN is probably crucial for the tissue injury in gouty arthritis [8], the influence of colchicine derivatives—demecolcine and deacetylcolchiceine—on lysosomal enzyme release from PMN after phagocytosis of monosodium urate crystals (MSU) and on the intracellular level of 3′, 5′ cyclic adenosine monophosphate (cAMP).

Demecolcine and deacetylcolchiceine were kindly supplied by Prof. F. Šantavý, M.D., Biochemical Institute of the Medical Faculty, Palacký University, Olomouc, Czechoslovakia.

MSU microcrystals (0.5–30 μm) were a gift from Dr. Továrek, University Hospital, Brno, Czechoslovakia.

Separation of leukocytes [9]. Leukocytes were obtained from the venous blood of healthy young men. Blood (450 ml) was drawn into plastic flasks with 0.9. ml of heparin (5000 U per ml) and 90 ml of a 6% dextran solution. Sedimentation was allowed to proceed in the same flasks for 40 min at room temperature. The cell-rich supernatant was sedimented at 100 *g* for 8 min at room temperature. The erythrocytes were removed by hypotonic lysis (90 ml 0.85% NaCl for 30 sec, 270 ml distilled water added for 20 sec, 90 ml 2.6% NaCl added, and the leukocytes were washed two more times in 0.15 M NaCl and resuspended in the buffered medium to a concentration of 5×10^7 leukocytes per ml medium (1% glucose in phosphate buffered saline, pH 7.4, containing 500 U of heparin per 100 ml.) Neutrophils were 60–75 per cent of total leukocytes.

Measurement of enzyme release. Portions of cell suspension 0.7 ml were dispensed into 10 × 75 mm plastic test tubes. The cells were incubated at 37° with gentle shaking with demecolcine or with deacetylcolchiceine in various concentrations 0.5 ml. Autologous serum was added to a concentration of 10%. After 1 hr incubation the cells were exposed for 1 hr to particles of microcrystalline monosodium urate 0.2 ml. The final concentration of urate was 0.5 mg per ml medium. At the end of experiments, tubes were centrifuged at 755 *g* at 4°. The cell-free supernatant fractions were used for enzyme determination. Portions (0.5 ml) of 0.05 M Tris–HCl buffer (pH 7.5) containing 4 mM EDTA were added to sediments (EDTA acts as a phosphodiesterase inhibitor to prevent degradation of cyclic nucleotides by plasma enzymes). Samples were heated to 100° and centrifuged at low speed. Supernatant fractions were stored at −20° for determination of cAMP.

Estimation of enzymatic activities. Lactate dehydrogenase (EC 1.1.1.27) was determined by the method of Bergmeyer *et al.* [10] and neutral proteases (EC 3.4.4) activity was estimated by the procedure reported by Ignarro [11].

Beta-glucuronidase (EC 3.2.1.31) activity was measured with phenolphthalein glucuronide as substrate [12] and acid phosphatase (EC 3.1.3.2) was established using *p*-nitrophenyl-phosphate as substrate [13].

Determinations of total enzyme activities were made after cells either incubated with tested drugs or without incubation were lysed by six freeze–thaw cycles. Broken cells preparations were centrifuged and enzyme activities were then determined. Incubation with added drugs did not influence the total enzyme activity in comparison with untreated cells.

The cyclic nucleotide levels were estimated by cyclic AMP assay kit (The Radiochemical Centre, Amersham, U.K.) [14, 15].

Polymorphonuclear leukocytes incubated with MSU crystals released lysosomal enzymes as well as cytoplasmic marker enzyme—LDH (Tables 1 and 2). The preincubation with both colchicine derivatives in various concentrations resulted in a decrease of lysosomal enzyme release. The inhibition was dose-dependent. The release of cytoplasmic enzyme LDH tended to decrease but not to such an extent as the release of lysosomal enzymes, especially in the experiment with demecolcine. Phagocytosis of MSU crystals induced the decrease of cAMP level (Table 3). In the presence of tested colchicine derivatives (in concentrations 10^{-3} and 10^{-4} M) there was an elevation in cAMP. This increase was dose-dependent.

The release of lysosomal enzymes may be influenced by several pharmacologic agents [16]. Three major types of compounds have been studied: (1) those that interfere with the levels of cyclic nucleotides—elevation of cAMP cellular levels reduces the enzyme release. Accumulation of cGMP results in enhancement of enzyme release from PMN; (2)

Table 1. The effect of demecolcine (DEME) on release of lysosomal enzymes*

	Lactate dehydrogenase (units)	Neutral protease (μg tyrosine/18 hr)	β-glucuronidase (μg phenolphthalein/18 hr)	Acid phosphatase (μg p-nitrophenol/hr)
Polymorphonuclear leukocytes (PMN)	151.3 ± 8.4 (6.2%)	453.02 ± 3.79 (7.5%)	94.66 ± 1.76 (5.3%)	177.30 ± 2.33 (9.9%)
PMN + urate (U)	426.5 ± 5.6 (17.5%)	2146.66 ± 8.51 (35.4%)	477.66 ± 8.02 (26.9%)	350.66 ± 4.09 (19.7%)
PMN + U + 10^{-3}M DEME	335.4 ± 7.2 (13.8%)	1067.00 ± 12.58 (17.6%)†	277.66 ± 3.84 (15.6%)†	207.21 ± 3.61 (11.6%)†
PMN + U + 10^{-4}M DEME	375.5 ± 4.9 (15.4%)	1574.33 ± 6.01 (25.9%)†	283.34 ± 2.03 (15.9%)†	253.33 ± 1.85 (13.2%)†
PMN + U + 10^{-5}M DEME	406.7 ± 9.6 (16.7%)	1848.50 ± 34.38 (30.5%)†	346.67 ± 2.40 (19.5%)†	290.31 ± 2.94 (16.3%)‡
Total activity (100%)	2433.9 ± 19.4	6065.10 ± 51.72	1778.33 ± 60.09	1782.66 ± 53.62

* Each number represents the mean of five samples. All activities were calculated for 5×10^7 cells. One unit of activity was defined as decrease of absorbancy 0.001 value per min at 340 nm at 25°.
† Statistically significant difference. $P < 0.01$.
‡ Statistically significant difference. $P < 0.02$.

Table 2. The effect of deacetylcolchiceine (DAC) on release of lysosomal enzymes*

	Lactate dehydrogenase (units)	Neutral protease (μg tyrosine/18 hr)	β-glucuronidase (μg phenolphthalein/18 hr)	Acid phosphatase (μg p-nitrophenol/hr)
Polymorphonuclear leukocytes (PMN)	181.4 ± 6.6 (7.8%)	214.68 ± 1.45 (3.4%)	101.05 ± 2.08 (7.7%)	168.23 ± 3.28 (9.2%)
PMN + urate (U)	343.7 ± 9.4 (14.8%)	1705.33 ± 23.32 (27.0%)	332.32 ± 3.28 (25.4%)	370.67 ± 4.76 (20.3%)
PMN + U + 10^{-3}M DAC	228.6 ± 7.1† (9.9%)	950.35 ± 8.11† (15.1%)	121.70 ± 8.82† (9.3%)	259.56 ± 8.80† (14.2%)
PMN + U + 10^{-4}M DAC	287.2 ± 7.7† (12.3%)	1310.67 ± 17.73† (20.7%)	197.05 ± 2.25† (15.1%)	344.32 ± 2.62† (18.9%)
PMN + U + 10^{-5}M DAC	321.7 ± 8.3‡ (13.8%)	1528.72 ± 9.12† (24.2%)	262.65 ± 2.03† (20.1%)	360.34 ± 4.45 (19.8%)
Total activity (100%)	2318.2 ± 33.4	6318.33 ± 4.41	1308.5 ± 41.60	1823.15 ± 41.63

* Each number represents the mean of five samples. All activities were calculated for 5×10^7 cells. One unit of activity was defined as decrease of absorbancy 0.001 value per min at 340 nm at 25°.
† Statistically significant difference. $P < 0.01$.
‡ Statistically significant difference. $P < 0.02$.

Table 3. The influence of demecolcine (DEME) and deacetylcolchiceine (DAC) on cyclic AMP levels*

	Cyclic AMP (pmoles/5 × 10^7 cells)
Polymorphonuclear leukocytes (PMN)	16.13 ± 0.26
PMN + urate (U)	15.47 ± 1.18
PMN + U + 10^{-3}M DEME	26.53 ± 0.61†
PMN + U + 10^{-4}M DEME	26.30 ± 1.14†
PMN + U + 10^{-5}M DEME	19.33 ± 0.71
Polymorphonuclear leukocytes (PMN)	13.73 ± 1.03
PMN + urate (U)	9.20 ± 0.55
PMN + U + 10^{-3}M DAC	16.27 ± 0.52†
PMN + U + 10^{-4}M DAC	14.96 ± 0.75†
PMN + U + 10^{-5}M DAC	9.87 ± 0.64

* Each number represents the mean of five samples.
† Statistically significant difference. $P < 0.01$.

agents which affect the state or function of cytoplasmic microtubules; (3) agents that are known to stabilize biomembranes.

Colchicine inhibits microtubule assembly both *in vitro* and *in vivo* by binding to tubulin with high affinity the major protein constitutent of microtubules. Malawista uses this mechanism for explanation of the fact that colchicine actively inhibits the extracellular release of lysosomal enzymes [17]. Colchicine also potentiates the increase of cAMP in human leukocytes induced by beta-adrenergic agonists and phosphodiesterase inhibitors [18].

The structure–activity relationship of colchicine derivatives has been investigated in several models. The results do not support the unitary hypothesis for colchicine activity, based on its interference with microtubular subunit protein aggregation. Deacetylcolchiceine, used in our study, is an active drug in acute gout, as in colchicine [19]. Deacetylcolchiceine has no effect on microtubular precursor protein and even in high concentrations has little or no antimitotic effect in a variety of cell systems *in vivo* and *in vitro* [20, 21]. Deacetylcolchiceine is nearly as effective as colchicine in inhibiting the PMN motility *in vitro* but [6] has no influence on the urate-induced paw swelling in mice [7]. Demecolcine, on the other hand, inhibits the urate-induced paw swelling in rat, as does colchicine [22, 23]. Demecolcine is about equipotent to colchicine in inhibiting the uptake of nucleoside in various mammalian cell lines [20]. Deacetylcolchiceine did not inhibit the nucleoside transport.

Conclusion. MSU crystals-induced release of lysosomal marker enzymes from PMN was inhibited by both colchicine derivatives—deacetylcolchiceine and demecolcine. Pretreatment of the cells with both drugs resulted in a intracellular elevation of cAMP. It seems that there is a certain correlation between the activity of these agents and the level of cAMP. This effect could be primary and may mediate later via the intracellular level of cyclic nucleotides the influence on the integrity of the microtubular system.

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