

COLCHICINE EFFECTS ON LYSOSOMAL ENZYME INDUCTION AND INTRACELLULAR DEGRADATION IN THE CULTIVATED MACROPHAGE*

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Previous studies have shown that endocytic stimuli result in increased lysosomal enzyme activity in the cultivated mouse peritoneal macrophage (1, 2). Inducers may be interiorized by phagocytosis or pinocytosis and may be as simple as L-amino acid polypeptides or as complex as serum or erythrocytes. A common feature of inducers is their susceptibility to degradation within secondary lysosomes. Indigestible substrates such as polystyrene particles or D-amino acid polypeptides are ineffective inducers (2).

Although the mechanisms for lysosomal enzyme induction are incompletely understood, it has been shown that colchicine inhibits enzyme induction by a pinocytic stimulus (3). In view of previous suggestions that the abundant arrays of microtubules within phagocytic cells are of importance in the promotion of fusion among endosomes and lysosomes (4-9), it was of interest to examine the possibility that colchicine inhibition of lysosomal enzyme induction was related to effects of the drug on microtubules and to inhibition of degradation of ingested substances. The effects of colchicine on induction of acid phosphatase by pinocytic and phagocytic stimuli and on degradation of endocytized materials are evaluated in this report.

Materials and Methods

Animals. Pathogen-free male white mice, 25-30 g, of the NCS/PA strain (10) were used in all experiments.

In Vitro Cultivation of Mononuclear Phagocytes. Cells were harvested from the peritoneal cavities of unstimulated mice in heparinized, phosphate-buffered saline, pH 7.4, by techniques previously described (1, 2). A 1.0 ml sample containing approximately 3×10^6 cells/ml in Medium 199 (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) containing 20% heat-inactivated newborn calf serum (NBCS)¹ (Grand Island Biological Co., Berkeley, Calif.) was dispensed to each 12 x 35-mm Leighton tube, without cover slips, and was incubated for 1 h at 37°C, after which the

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¹ Abbreviations used in this paper: BSS, balanced salt solution; CFU, colony-forming units; [³H]ABA, [³H]alpha aminoisobutyric acid; NBCS, newborn calf serum; PBS, phosphate-buffered saline.

adherent cells were washed twice with Medium 199. The monolayers were then reincubated in Medium 199 containing 30% NBCS and potassium penicillin G (1,000 U/ml) (Eli Lilly & Co., Indianapolis, Ind.). Penicillin G was incorporated in fresh medium at all medium changes. When experimental design required cultivation of the monolayers for longer than 24 h, the medium was changed daily. Unless otherwise specified, all cultivation medium consisted of Medium 199 with 30% NBCS and penicillin G (1,000 U/ml).

Inhibitors. Colchicine (Sigma Chemical Co., St. Louis, Mo.) was made up as 10^{-1} or 10^{-3} M stock solutions in phosphate-buffered saline, pH 7.4, and stored frozen as 0.5-ml aliquots. Lumicolchicine, a mixture of photoisomers of colchicine (11), was kindly prepared by Leslie Wilson, Department of Pharmacology, Stanford University School of Medicine, by ultraviolet irradiation of an ethanol solution of colchicine. After vacuum evaporation of the alcohol, the residue was reconstituted to a 10^{-3} M solution in phosphate-buffered saline, pH 5.0, and stored at -20°C in 0.5-ml portions. Purity of the preparation was confirmed by its absorption spectrum and lack of tubulin-binding properties.

Cycloheximide (Sigma Chemical Co.) was used as an inhibitor of protein synthesis. Solutions were freshly made each day.

Acid Phosphatase Assay. At the time of harvest of cells which were to be analyzed for enzyme content the medium was decanted from duplicate Leighton tubes and the monolayers rinsed three times with normal saline. Each tube then received 0.5 ml normal saline and was stored at -20°C until the time of assay. Cells were separated from the glass by subjecting the tubes to four freeze-thaw cycles and scraping with a rubber policeman. Assay for acid phosphatase activity based on the hydrolysis of α -naphthol acid phosphate using methods previously described (12) was performed on 0.1-ml aliquots. Enzyme levels were determined by measuring increase in absorbance at 545 nm in a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

All enzyme activities are corrected for cell protein and are expressed as nanomoles inorganic phosphate released \times minutes $^{-1}$ \times milligrams $^{-1}$ cell protein. This expression of activity will be considered to represent acid phosphatase specific activity.

Protein Assay. Protein content was determined by the method of Lowry (13) using crystalline egg white lysozyme as the standard.

Sucrose Uptake. Macrophages which had been allowed to mature in 30% NBCS for 24 h were rinsed with Medium 199 and incubated in fresh Medium 199 containing 50, 30, or 1% NBCS, with or without 10^{-6} M colchicine or 10^{-5} M lumicolchicine. After an additional 2 h incubation, 0.05 ml of 0.6 M sucrose containing 5.0 μCi [^3H]sucrose (glucose-D-[1- ^3H]fructose, sp act 4.79 Ci/mmol, New England Nuclear, Boston, Mass.) was added to each tube. Duplicate samples from all experimental groups were harvested at 2, 4, and 6 h after addition of the radiolabel. All tubes were rinsed four times with 5-ml vol of Medium 199 containing 0.03 M sucrose and the monolayers dissolved in 0.5 ml of 0.1 N NaOH. Aliquots of the hydrolysate were assayed for protein as described above. 0.25-ml aliquots were transferred to scintillation vials and mixed with 10 ml scintillation fluid (5.76 ml toluene, 0.24 ml Liquifluor [New England Nuclear], and 4.0 ml ethylene glycol monomethyl ether). A Mark II liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.) was used to assay for radioactivity.

[^3H]Sucrose Degradation. Macrophage monolayers that had been allowed to mature for 24 h in Medium 199 containing 30% NBCS received fresh Medium 199-30% NBCS with the addition of 4.5 $\mu\text{Ci}/\text{ml}$ [^3H]sucrose with 0.03 M unlabeled carrier sucrose. After an additional 24 h of incubation at 37°C , sucrose-containing medium was removed and the monolayers rinsed four times with Medium 199. Fresh Medium 199-30% NBCS was added to control tubes and colchicine (10^{-6} - 10^{-3} M) was added to remaining tubes. After incubation for an additional 2 h at 37°C , duplicate tubes from control and colchicine groups were harvested and designated as 0 time samples. Invertase (melibiase-free, Nutritional Biochemicals Corp., Cleveland, Ohio) in varying concentrations was then added and duplicate samples from both control and colchicine-treated cells were harvested hourly during the subsequent 6 h. Control and colchicine-treated cells to which invertase was not added were also harvested.

Monolayers were harvested by rinsing four times with normal saline (5 ml) after which 0.5 ml of 0.5 N NaOH was added to digest cell protein. Aliquots were assayed for protein and for radioactivity as described above.

Bacterial Uptake and Degradation. *Staphylococcus albus* and *Bacillus subtilis* were used for studies of bacterial uptake and degradation. A single colony of *S. albus* or 0.01 ml of a spore

suspension of *B. subtilis* (approximately 10^9 spores/ml) was inoculated into a medium composed of 48 ml leucine assay medium (Difco Laboratories, Detroit, Mich.), 2 ml Medium 199, and 0.2 mCi [^3H]leucine (L-[4,5- ^3H ,N]leucine, sp act 33.6 Ci/mmol, New England Nuclear). The bacteria were incubated for 18–24 h at 37°C in a shaking water bath, after which the bacteria were sedimented by centrifugation at 12,000 *g* for 10 min. The sediment was washed 4 times with sterile phosphate-buffered saline (PBS), pH 7.4, aliquots were taken for estimation of colony count by plate-counting technique, and the bacterial suspension was killed by autoclaving for 15 min. After sterilization, the suspension was again rinsed four times with PBS. After repeated rinsings, less than 5% of the total radiolabel was found in the TCA-soluble fraction of the bacterial suspension.

24-h old monolayers were used in all experiments involving bacteria. Colchicine (10^{-6} M) was added to the cells in the treatment group 2 h before addition of bacteria and was present for the duration of the experiments. The bacterial inoculum was in the range of $2-4 \times 10^6$ colony-forming units (CFU)/ml, or approximately 20–40 CFU/mononuclear phagocyte. For evaluation of uptake, duplicate tubes from control and colchicine groups were harvested at hourly intervals after the addition of particles. At the time of harvest, the medium was decanted and frozen, and the tubes were rinsed four times with 5-ml portions of normal saline. Each tube was frozen with a final aliquot of 0.5 ml normal saline. Monolayers were removed from the glass and the cells disrupted by freeze-thaw treatment as described above. 0.25-ml aliquots of the cell suspension and 0.5-ml aliquots of the medium were precipitated with 7% TCA at 0–4°C. 0.25-ml aliquots of the TCA-soluble fraction were transferred to scintillation vials. The precipitates were washed twice with 7% TCA, dissolved in 0.5 N NaOH and transferred to scintillation vials. Scintillation fluid and counting for radioactivity were as described above. Accumulation of radiolabel in the TCA-precipitable fraction of the monolayer was considered to represent ingestion.

For evaluation of degradation, monolayers were allowed to ingest the bacteria for 1 or 2 h. Uningested bacteria were removed by aspiration of medium and rinsing four times with Medium 199. Fresh medium was added and incubation was continued. Duplicate tubes from both experimental and control groups were harvested at intervals during the following 24 h and processed in the same manner as in evaluation of ingestion. Conversion of the radiolabel from that associated with the TCA-precipitable monolayer protein to that associated with the TCA-soluble fraction of the medium was considered to represent degradation of bacterial protein (14).

There was less intertube variation in total radiolabel counts in the experiments involving *S. albus* than in those involving *B. subtilis*. For this reason, the degradation of *S. albus* is expressed as percentage of the radiolabel in the TCA-precipitable fraction of the monolayer at the end of the phagocytic pulse which had been converted to TCA-soluble label in the medium at each indicated time, while the degradation of *B. subtilis* is expressed as percentage of the total counts in the tube which are associated with the TCA-soluble fraction of the medium at each indicated time.

Protein Synthesis. The incorporation of [^3H]leucine into TCA-precipitable cell protein by macrophage monolayers was used to estimate protein synthesis. Serum-containing medium was rinsed from 24-h old monolayers with three washes of leucine-free Medium 199, and the cells were cultivated for 2 h in leucine-free Medium 199 without added serum. Inhibitors were added at the beginning of cultivation in leucine-free medium. 5.0 μCi [^3H]leucine was added to each tube and cultivation at 37°C was continued. Duplicate samples in each experimental group were harvested at 60 min. Preliminary experiments had indicated that, under the conditions employed, uptake of [^3H]leucine was linear for at least 60 min in all groups. The medium was removed and the monolayers were rinsed four times with normal saline, covered with 0.5 ml normal saline, and frozen. Cells were harvested by freeze-thaw cycles and scraping, and aliquots precipitated in 7% TCA, as described above. Only the radioactivity in the TCA-precipitable fraction of the cell protein was assayed. Uptake of [^3H]leucine was corrected for total cell protein.

Amino Acid Uptake. Uptake of the nonassimilable amino acid, [^3H]alpha aminoisobutyric acid, ([^3H]ABA) using slight modifications of published techniques (15), was used as an index of amino acid uptake by macrophages.

24-h old monolayers were rinsed with Earle's balanced salt solution (BSS) (Microbiological Associates, Inc., Bethesda, Md.), inhibitors were added, and the monolayers were again incubated at 37°C for approximately 90 min in BSS. 5.0 μCi [^3H]ABA (sp act 2.5 Ci/mmol) (ICN, Isotope and Nuclear Division, Irvine, Calif.) was added and duplicate tubes were harvested from each experimental group at 0, 5, 10, 15, and 30 min after addition of radiolabel. Uptake of radiolabel was

halted and free label removed by rapidly rinsing the tubes four times with 12-ml vol of iced normal saline. Monolayers were then digested with 0.5 ml of 0.5 N NaOH. 0.25 ml of the final solution was assayed for radioactivity as described above. Uptake of [^3H]ABA is expressed as nanomoles [^3H]ABA per microgram cell protein.

Morphology. When detailed microscopic examination of monolayers was desired and for photomicrography, preparation of monolayers differed from the method described above only in that cover slips were used in the Leighton tubes. The medium was decanted and cells were fixed with glutaraldehyde at 4°C.

Data Analysis. With few exceptions, which are clearly indicated, all data are expressed as the mean \pm SE of duplicate values from three or more experiments. Statistical inferences are derived for Student's *t* test for unpaired samples. Only $P \leq 0.05$ (two-tailed) was considered to be statistically significant. Straight lines, when shown, represent least mean squares analyses of the data.

Results

Cell Morphology. Addition of colchicine at concentrations of 10^{-6} M or greater caused macrophages cultivated in Medium 199 with 30% NBCS to become rounded within 2 h. Over the following 6–8 h, the cells began to assume bizarre forms as previously described by Bhisey and Freed (16, 17). By 24 h all cells were clearly abnormal. The fraction of cells with obviously abnormal form at 24 h after addition of colchicine increased as the concentration of colchicine in the medium was increased from $10^{-7.5}$ to 10^{-6} M, with approximately one-half the cells rendered abnormal by 10^{-7} M colchicine. Increasing the concentration of the drug in the range of 10^{-6} – 10^{-3} M did not further alter the appearance of individual cells. However, the initial rounding seemed to occur more rapidly with higher concentrations of the drug, an effect that has been observed by others (16), and there was increased detachment of cells from the glass.

Morphological changes produced by cultivating macrophages in 10^{-6} M colchicine for 24–48 h were fully reversible. Within 24 h after removal of the drug, colchicine-treated cells were indistinguishable from controls. Lumicolchicine, a mixture of photoisomers of colchicine which retains the less specific membrane effects of colchicine while lacking its tubulin-binding properties (18), did not alter the appearance of macrophages even when used at concentrations as high as $10^{-4.5}$ M.

Acid Phosphatase. Although previously noted (3), the effects of colchicine on acid phosphatase induction have not been well characterized. The rise in specific activity of acid phosphatase resulting from cultivation of macrophages in medium containing 30% NBCS, which occurred between 24 and 72 h of cell age after explant, and the complete inhibition of this rise by 10^{-6} M colchicine are illustrated in Fig. 1.

The inhibitory effect of colchicine on acid phosphatase induction by pinocytosis paralleled the action of the drug on alterations in cell shape. Maximal inhibition of acid phosphatase induction was attained at concentrations of colchicine (10^{-6} M) which caused abnormal shapes to develop in all the cultured cells. Further increases in colchicine concentration did not significantly enhance inhibitory activity. Furthermore, at concentrations below $10^{-7.5}$ M, colchicine was ineffective as an inhibitor of lysosomal enzyme induction by pinocytosis, while concentrations intermediate between the extremes resulted in intermediate inhibition of acid phosphatase induction (Fig. 2).

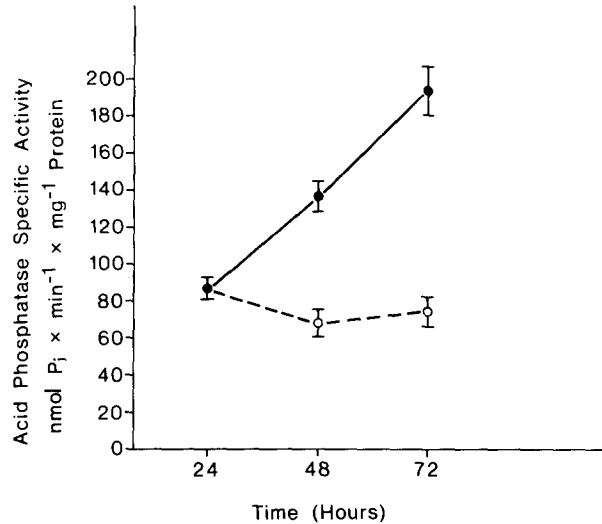


FIG. 1. Temporal change in acid phosphatase specific activity for control (●) and colchicine-treated (10^{-6} M) (○) macrophages cultivated in 30% NBCS. Mean \pm SE of duplicate values from six experiments.

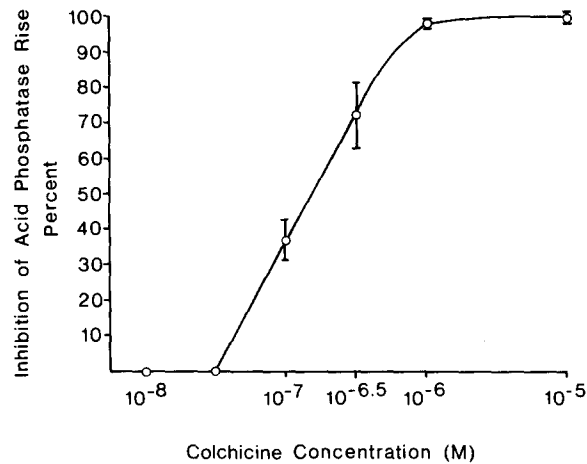


FIG. 2. Dose-response curve for inhibition of acid phosphatase induction by colchicine. Mean \pm SE of duplicate values from three experiments.

To further examine the possibility that both morphological changes and inhibition of enzyme induction were related to colchicine's action on microtubules, macrophages cultivated in 30% serum as above were exposed to lumicolchicine in concentrations ranging from 10^{-6} to 10^{-4} M. No effect of this drug on lysosomal enzyme induction could be demonstrated, and acid phosphatase levels were indistinguishable from those in controls after cultivation of the cells in $1-3 \times 10^{-5}$ M lumicolchicine for 48 h ($93.8 \pm 4.7\%$ of control levels).

Because inhibitors of pinocytosis also inhibit lysosomal enzyme induction resulting from cultivation of macrophages in serum (3), the effects of colchicine

on pinocytosis, as measured by uptake of sucrose (19, 20), were evaluated. Accumulation of sucrose 6 h after addition of the tritiated sugar and the influence of varying serum concentrations, colchicine (10^{-6} M), and lumicolchicine (10^{-5} M) are illustrated in Fig. 3. Colchicine significantly retarded sucrose uptake in medium containing 30% and 50% NBCS, but did not significantly inhibit the ability of cells cultivated in 1% NBCS to accumulate the sugar. In contrast, lumicolchicine did not significantly retard sucrose uptake in 50% NBCS. Also illustrated in Fig. 3 is the effect of serum concentration on sucrose uptake. In the absence of colchicine, sucrose uptake increased in a proportional and nearly linear fashion with serum concentrations over the range from 1–50% NBCS. In

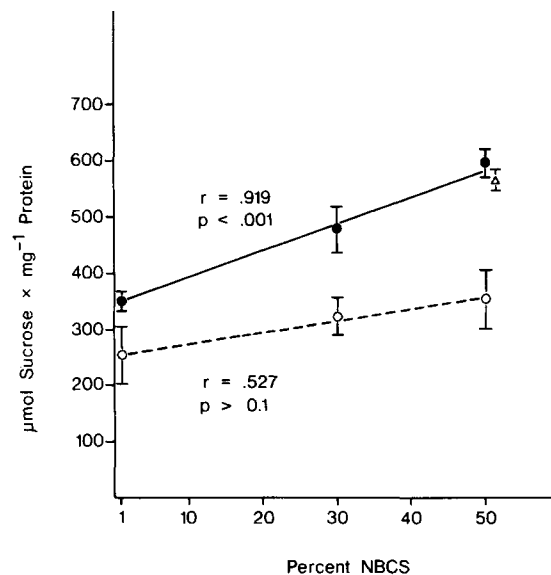


FIG. 3. Uptake of [^3H]sucrose in 0.03 M unlabeled sucrose; effect of varying serum concentrations for control (●), 10^{-6} M colchicine-treated (○), and $10^{-4.5}$ M lumicolchicine-treated (Δ) macrophages. The slopes of the regression lines for colchicine-treated and control macrophages differ significantly ($P < 0.05$). Mean \pm SE of duplicate values from three experiments.

the presence of colchicine (10^{-6} M), sucrose uptake in 50% NBCS was not significantly different than that in 1% NBCS. These data suggest that colchicine is a potent inhibitor of pinocytosis by the cultivated macrophage.

If the inhibition of pinocytosis were the primary or sole mechanism underlying colchicine inhibition of acid phosphatase induction, the drug would be expected to have no effect on lysosomal enzyme induction by a phagocytic stimulus. The effect of colchicine on acid phosphatase induction by ingested sheep erythrocytes was therefore examined.

Control cells that had been allowed to mature for 24 h were cultured an additional 24 h in Medium 199 and 30% NBCS. Another group of cells was cultivated in Medium 199 and 1% NBCS after rinsing the monolayers with Medium 199. A phagocytic stimulus was provided by addition of aldehyde-treated sheep erythrocytes (SRBC) (Difco Laboratories, Detroit, Mich.) ($3 \times 10^6/\text{ml}$) to the 24-h old

cells. The phagocytic stimulus was terminated after 1 h by aspiration of the medium and rinsing of the monolayers three times with Medium 199, after which the appropriate medium with 30% or 1% NBCS was added. One group of cells that was to be exposed to 1% NBCS and colchicine (10^{-6} M) received the combination immediately after termination of the phagocytic pulse. Another group exposed to 1% serum received colchicine 2 h before the addition of SRBC. Colchicine was added to cells which were cultivated without SRBC at the time of the medium change. The time at which SRBC were added was considered to be time zero and experiments were terminated 24 h later.

Fig. 4 illustrates that both 30% NBCS and SRBC with 1% NBCS induced acid phosphatase activity in the 24-h period of cultivation, while 1% NBCS was

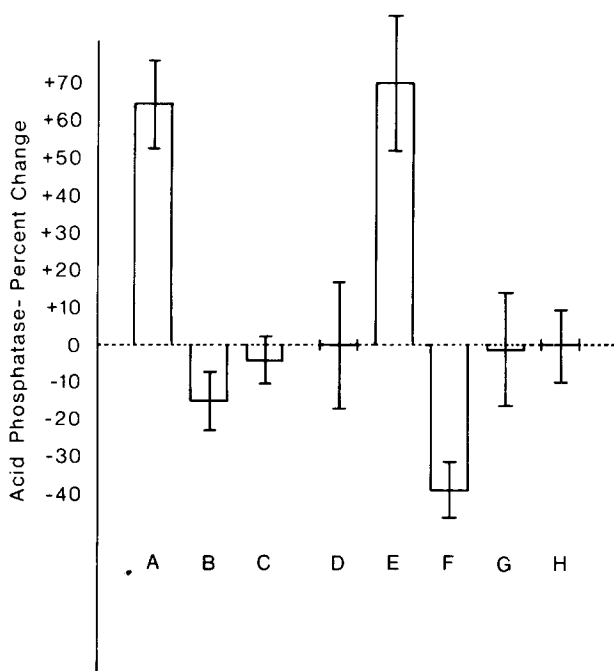


FIG. 4. Effect of colchicine on acid phosphatase (AP) induction by phagocytic and pinocytic stimuli. Cells cultivated in 1% or 30% NBCS, with or without SRBC, with or without colchicine (10^{-6} M). (A), 30% NBCS; (B), 30% NBCS-colchicine; (C), 30% NBCS plus SRBC**^{*}-colchicine; (D), 1% NBCS; (E), 1% NBCS plus SRBC**^{*}; (F), 1% NBCS-colchicine; and (G), 1% NBCS plus SRBC**^{*}-colchicine; and (H), 1% NBCS plus SRBC*^{*}-colchicine. SRBC were added either 1 h before (*) or 2 h after (**) colchicine.

$$\% \text{ change} = 100 \left(\frac{\text{AP specific activity 48 h} - \text{AP specific activity 24 h}}{\text{AP specific activity 24 h}} \right)$$

Mean \pm SE of duplicate values from four experiments.

ineffective. Colchicine (10^{-6} M) completely inhibited the rise in acid phosphatase activity whether the inducer was a pinocytic (B), phagocytic (G, H), or a combination of both (C) stimuli. Moreover, the inhibition of acid phosphatase induction by colchicine was complete whether the ingestion of SRBC occurred before (G) or after (H) the cells were treated with colchicine.

These results indicate that colchicine effectively inhibited the induction of acid phosphatase activity by both phagocytic and pinocytic stimuli, and that the inhibition results from interference in the processes responsible for induction at some step that occurs after particles have been interiorized. Because the morphological effects of colchicine were found to be reversible within 24 h after the drug was removed from the culture medium it was of interest to determine if the inhibitory effect on acid phosphatase induction was similarly reversible.

Colchicine (10^{-6} M) was added to macrophages 3-4 h after explantation in order that colchicine could be removed from the cells at a time during which control cells were still rapidly increasing in enzyme content. Four experimental groups were utilized: no colchicine, colchicine present for 96 h, colchicine removed at 24 h, and colchicine removed at 48 h. All cells were cultivated in Medium 199 with 30% NBCS.

Inhibitory effects of 24 or 48 h of colchicine exposure on acid phosphatase induction by cultivation in 30% NBCS were reversed within 24 h after removal of the drug (Fig. 5). Although the acid phosphatase specific activity in cells after removal of colchicine was at all time points significantly lower than control levels, the activities were significantly higher than those in cells with continuing exposure to the drug. The rate of increase of enzyme levels in cells released from the inhibition of colchicine approximated that in control cells.

Protein Synthesis. Colchicine is not generally thought to be an inhibitor of protein synthesis (21). However, there are data suggesting that, in some cell lines (22), the drug may interfere with protein synthesis, and there is no specific mention concerning its effects on this function in macrophages. For this reason

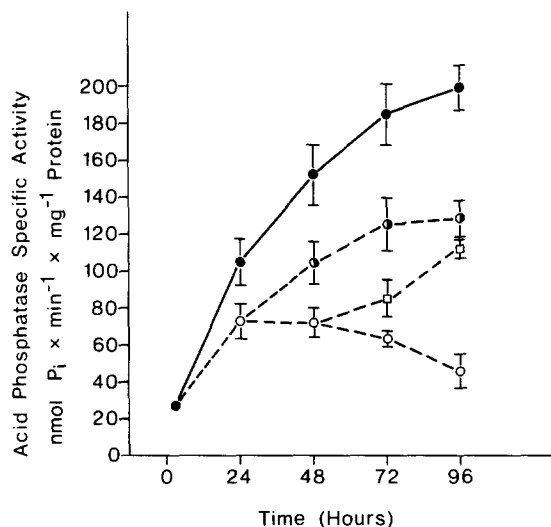


FIG. 5. Reversal of inhibition of acid phosphatase induction after removal of colchicine (10^{-6} M). Temporal change in acid phosphatase specific activity for control cells (●), cells exposed to colchicine for duration of experiment (○), cells exposed to colchicine from 4 to 24 h (●), and cells exposed to colchicine from 4 to 48 h (□). Mean \pm SE of duplicate values from four experiments.

the possibility that colchicine's inhibition of lysosomal enzyme induction might be mediated by an inhibitory effect on protein synthesis was examined.

Colchicine ($\geq 10^{-6}$ M) inhibited the accumulation of [3 H]leucine into TCA-precipitable macrophage protein by 25% compared with controls under the conditions used in these experiments (Fig. 6). In contrast, lumicolchicine had no effect on protein synthesis. Additionally, the effect of colchicine was not enhanced by increasing the concentration of colchicine beyond 10^{-6} M. The

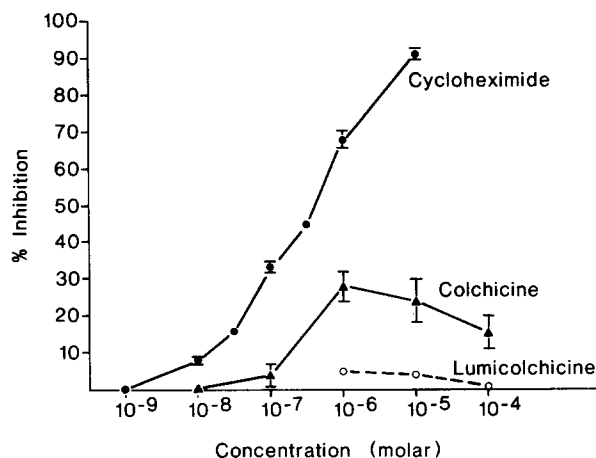


FIG. 6. Inhibition of incorporation of [3 H]leucine into TCA-precipitable cell protein by cycloheximide (●), colchicine (▲), and lumicolchicine (○).

$$\% \text{ inhibition} = 100 \times 1 - \left(\frac{\text{cpm/mg cell protein [inhibitor]}}{\text{cpm/mg cell protein [control]}} \right)$$

Mean \pm SE of duplicate values from four experiments.

degree of inhibition of protein synthesis produced by colchicine was comparable to that produced by very low concentrations of cycloheximide (10^{-8} – 10^{-7} M). These concentrations of cycloheximide did not alter acid phosphatase induction resulting from cultivation of macrophages in 30% NBCS for 48 h ($107 \pm 6.5\%$ of control levels), although 10^{-6} M cycloheximide (70% inhibition of protein synthesis) did inhibit enzyme induction.

Since, under certain circumstances, colchicine has been shown to inhibit lysine transport in rabbit phagocytes (23), the measured inhibition of incorporation of [3 H]leucine into TCA-precipitable protein could have been a reflection of inhibition of amino acid uptake rather than a specific effect on protein synthesis. Therefore, uptake of the nonassimilable amino acid [3 H]ABA was examined to discriminate drug-induced alterations in uptake from effects on cellular protein synthesis (15).

Colchicine (10^{-6} M) retarded the uptake of [3 H]ABA, while lumicolchicine exerted no effect (Fig. 7). Moreover, the uptake of [3 H]ABA was linear for the initial 30-min time period for control and lumicolchicine-treated cells but was clearly nonlinear for colchicine-treated cells.

Degradation of Intracellular Sucrose by Exogenous Invertase. The role of

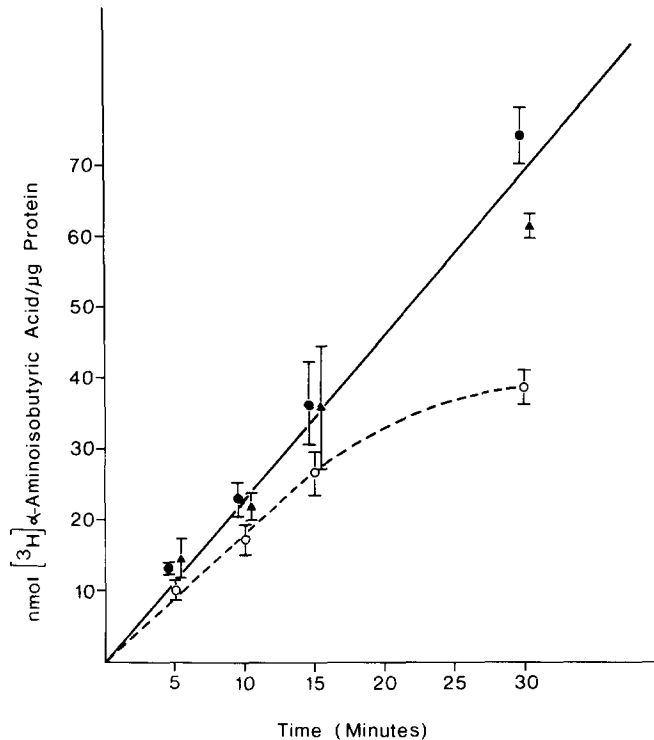


FIG. 7. Uptake of [^3H]aminoisobutyric acid by macrophages. Effect of 10^{-6} M colchicine (O) (three experiments) and 10^{-5} M lumicolchicine (\blacktriangle) (two experiments) compared with control (\bullet) (three experiments). Mean \pm SE (colchicine, control) or mean \pm range (lumicolchicine) of duplicate values.

microtubules in the fusion of lysosomes with newly ingested pinosomes was evaluated by measuring the effect of colchicine on degradation of intracellular sucrose by added invertase. Since macrophages lack enzymes with invertase activity, nonhydrolyzed sucrose accumulates within pinolysosomes which swell, presumably secondary to the accumulation of water across an osmotic gradient. Invertase, added to the medium, is also interiorized by pinocytosis. These pinocytic vesicles would be expected to fuse with the previously formed secondary lysosomes; when the latter contain sucrose, the sugar is hydrolyzed to its diffusible monosaccharide constituents (19).

Macrophages which had been allowed to accumulate [^3H]sucrose with 0.03 M unlabeled carrier sucrose for 24 h contained large numbers of phase lucent vacuoles. When the medium was changed to sucrose-free medium, the cell-associated radiolabel remained quite stable, such that approximately 10% was lost over a 6 h period. Addition of invertase (375 $\mu\text{g}/\text{ml}$) resulted in accelerated loss of radiolabel as the sucrose was hydrolyzed to glucose and [^3H]fructose, and the latter diffused into the medium. The loss of radiolabel proceeded linearly during the 6-h experimental period, at the end of which invertase-treated cells had approximately 25% the intracellular sucrose concentration of control cells incubated without invertase. At all time points, loss of radiolabel was unaffected

by pretreatment of cells with colchicine (10^{-6} M) for 2 h before addition of invertase. Increasing the concentration of colchicine to 10^{-3} M was similarly without effect, as was increasing the duration of exposure of the cells to colchicine before addition of invertase (4 or 24 h). To examine the possibility that the concentration of invertase in the medium was so excessive as to mask any effect of colchicine on sucrose hydrolysis, a wide range of concentrations of invertase, from 0.6 $\mu\text{g/ml}$ through 375 $\mu\text{g/ml}$ was studied. Loss of radiolabel from the monolayers was directly proportional to the concentration of added invertase (Fig. 8), and was unaffected by pretreatment of the cells with colchicine (10^{-6} M).

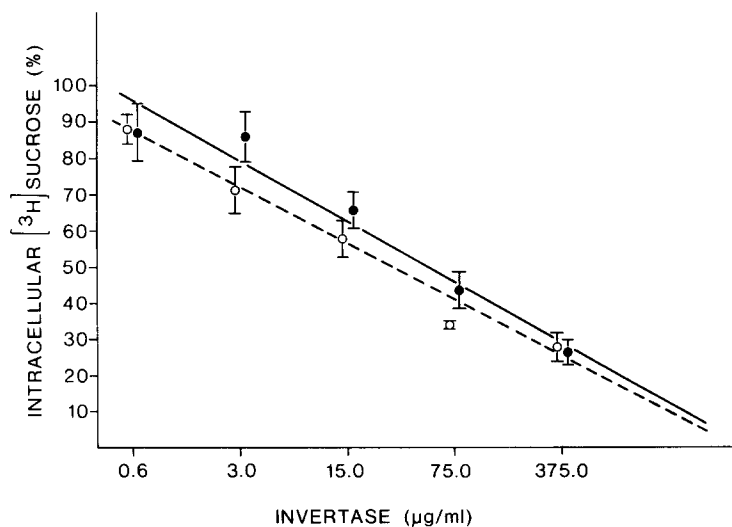


FIG. 8. Dose-response relationship of varying concentrations of exogenous invertase on loss of [^3H]sucrose from macrophages. Comparison of control (●) with cells treated with 10^{-6} M colchicine (○).

$$\% \text{ intracellular } [^3\text{H}] \text{ sucrose} = 100 \left(\frac{\text{cpm/mg protein [invertase-treated] at 6 h}}{\text{cpm/mg protein [no invertase] at 6 h}} \right).$$

Mean \pm SE of duplicate values from six experiments.

Bacterial Degradation. Although no effect of colchicine on degradation of sucrose by exogenous invertase, and by inference no effect of colchicine on fusion among pinosomes and secondary lysosomes, was demonstrated, it remained possible that, with a different substrate, inhibition could be demonstrated. To further examine this possibility, the degradation of radiolabeled bacteria by control and colchicine-treated macrophages was evaluated. Data for uptake and degradation of [^3H]leucine-labeled *S. albus* and *B. subtilis* are presented.

Colchicine (10^{-6} M) exerted no measurable effect on uptake of either bacterial species. This was evident if only the radiolabel present in undegraded bacterial protein (TCA-precipitable monolayer fraction) was considered (Fig. 9 a) or if the products of early degradation (TCA-soluble medium fraction) were added (Fig. 9 b). Similarly, the drug did not alter intracellular degradation of radiolabeled

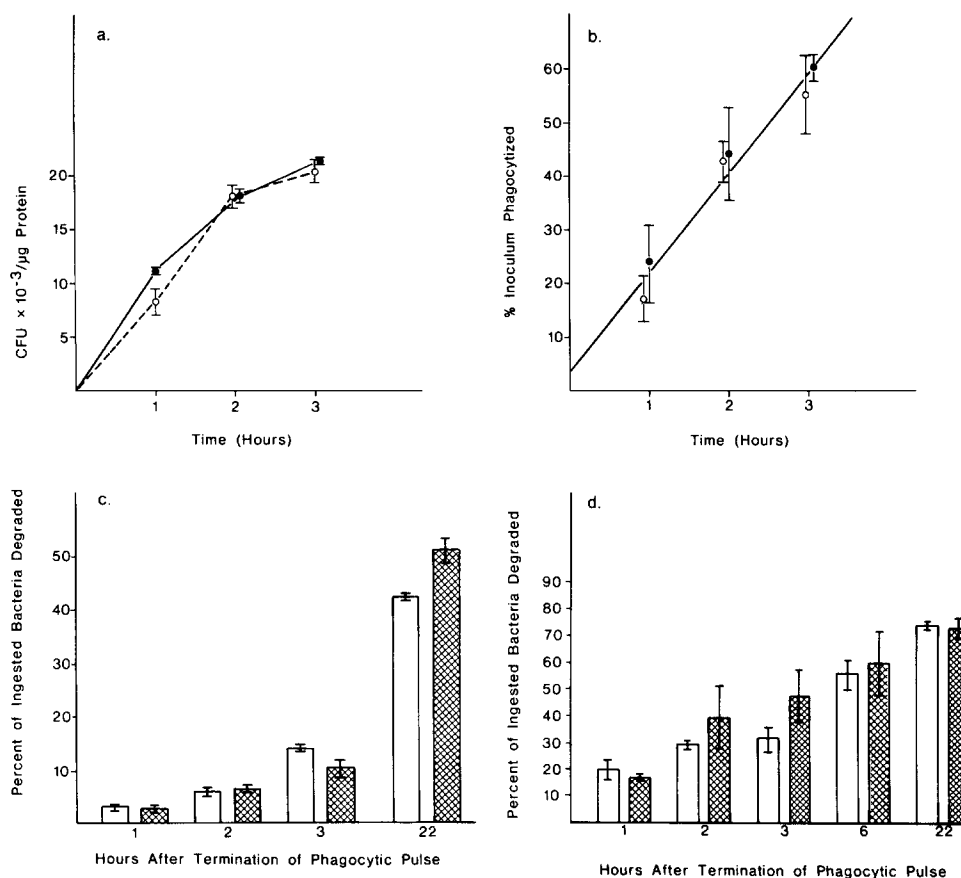


FIG. 9. Lack of colchicine (10^{-6} M) effect on uptake and degradation of [3 H]leucine-labeled heat-killed bacteria. Uptake of *S. albus* (a) normalized for total monolayer protein and of *B. subtilis* (b) by control (●) and colchicine-treated (○) macrophages. Degradation of ingested *S. albus* (c) and *B. subtilis* (d) by control (open bars) and colchicine-treated (cross-hatched bars) macrophages.

$$\% \text{ degraded} = 100 \left(\frac{\text{cpm in TCA-soluble medium}}{\text{total cpm in tube}} \right) \text{ at indicated time.}$$

Mean \pm SE of duplicate values from three experiments.

bacterial protein of either *S. albus* or *B. subtilis* by mouse peritoneal macrophages cultivated in vitro (Figs. 9 c and 9 d).

Discussion

These studies were performed to evaluate the hypothesis that microtubules are of functional significance in induction of lysosomal enzymes and in intracellular degradation of materials endocytosed by phagocytic cells. Colchicine was chosen as an agent with known ability to disrupt microtubules through its ability to bind with and prevent polymerization of tubulin (24). The shape and spontaneous motility of macrophages are altered by the drug (16, 17) and the action of migra-

tion inhibitory factor on guinea pig macrophages is inhibited by concentrations as low as 10^{-7} M (25).

In addition, colchicine has been shown to inhibit the *in vitro* induction of certain lysosomal enzymes in the maturing mouse macrophage (3). Axline and Cohn (2) have shown that only materials that are digestible by the peritoneal macrophage are effective inducers of lysosomal enzymes and others have suggested that microtubules are necessary for the fusion of lysosomes with endosomes which must precede intracellular digestion of ingested materials (4-9, 26, 27). If microtubules do play such a role, colchicine could inhibit lysosomal enzyme induction by inhibiting lysosomal fusion and rendering ordinarily digestible materials indigestible.

Our results indicate that colchicine reversibly inhibits induction of acid phosphatase resulting from either a phagocytic or pinocytic stimulus. This effect on enzyme induction was evident at the same concentrations of colchicine which caused morphologic alterations indicative of microtubular depolymerization (16, 28) in macrophages.

Additional evidence obtained through use of lumicolchicine suggests that the factor underlying the above phenomena was disruption of microtubules (18). Photoinactivation of colchicine yields lumicolchicine which, lacking tubulin-binding properties, is incapable of depolymerizing microtubules and is without antimetabolic (29) and anti-inflammatory (30) effects, although it retains colchicine's effects on membrane transport systems (18). In our experiments, lumicolchicine, at concentrations 100 times greater than effective molar concentrations of colchicine, did not alter cell morphology or inhibit lysosomal enzyme induction.

Deuterium oxide has been shown in other systems to stabilize the microtubular network (31) and to antagonize the action of colchicine on histamine secretion by leukocytes (27, 32). In separate experiments not outlined above, exposure of cells to deuterium oxide at final concentrations up to 54% did not antagonize colchicine's effects on cell form or lysosomal enzyme induction in the macrophage.

It is unlikely that inhibition of pinocytosis is the primary basis for colchicine's inhibition of lysosomal enzyme induction. Cultivation of peritoneal macrophages in serum-poor medium, which does not provide a pinocytic stimulus, does not induce lysosomal enzymes. However, the lack of enzyme induction is readily overcome by phagocytic stimuli (2). A brief phagocytic pulse (of aldehyde-treated SRBC in our experiments) resulted in lysosomal enzyme induction comparable to that found secondary to cultivation in medium containing 30% serum. Inhibition of induction by colchicine differed from lack of induction resulting from cultivation in serum-poor medium in that the phagocytic pulse did not overcome colchicine's inhibition of enzyme induction.

Although inhibition of RNA synthesis by colchicine has been tentatively advanced as a mechanism for inhibition of lysosomal enzyme induction (3), the drug is not now thought to effect RNA synthesis. It does inhibit purine and pyrimidine uptake, but this uptake is not essential for normal nucleotide synthesis in mammalian cells (18).

Similarly, colchicine's inhibition of [3 H]leucine incorporation into TCA-

precipitable material by macrophages seems unlikely to account for the drug's effects on acid phosphatase induction. Inhibitors of protein synthesis are known to inhibit lysosomal enzyme induction (3) and cycloheximide was shown to inhibit acid phosphatase induction in our experiments. However, only when the concentration of cycloheximide was increased to levels which inhibited protein synthesis by 70% (10^{-6} M) did cycloheximide inhibit induction of acid phosphatase by pinocytotic stimuli. Colchicine did not inhibit [3 H]leucine incorporation by more than 25%, but completely inhibited acid phosphatase induction resulting from cultivation of macrophages in 30% NBCS between 24 and 96 h after explant.

On the basis of the inhibition of [3 H]ABA uptake by colchicine, we conclude that the drug inhibits amino acid uptake in this cell type and do not postulate a separate effect of the drug on protein synthesis per se. Colchicine has been shown to inhibit lysine incorporation by rabbit phagocytes under certain conditions (23); others (18) have found no effect of the drug on amino acid uptake in a variety of cell types.

The lack of effect of lumicolchicine on uptake of [3 H]leucine and [3 H]ABA suggests that amino acid uptake by the mouse peritoneal macrophage may require participation of intact microtubules. Alternatively, just as colchicine and lumicolchicine exhibit differences in binding to mammalian cell membranes (33, 34), these two agents could produce differential effects on certain membrane-mediated functions.

Although colchicine effects on acid phosphatase changes in vitro may be related to an interaction of the drug with microtubules it is unlikely that these effects are explicable by inhibition of fusion among lysosomes and endosomes and consequent inhibition of degradation of endocytized substances. No effect of colchicine on degradation of intracellular sucrose by exogenous invertase or on degradation of bacteria ingested by macrophage monolayers could be shown. However, it remains possible that when intact microtubules are present they are of functional significance in the promotion of fusion of endosomes with lysosomes. It would then be necessary to assume that the churning, ameboid movements which have been described in colchicine-treated macrophages (16, 17) have increased random contact among subcellular particles, thus negating the expected inhibitory effect of colchicine.

Several groups have attempted to define the role of microtubules in lysosomal fusion in phagocytic cells (6-9, 17, 27, 35-37). Weissmann et al. (6) have noted that protein degradation was retarded by treatment of macrophages with colchicine. Other authors have based their conclusions on the loss of lysosomal enzymes into the medium which occurs consequent to phagocytosis of inert particles (7), which follows attempted phagocytosis of biologically active materials that are too large to be interiorized (8, 9, 35), or which results from treatment of phagocytes with cytochalasin B followed by a phagocytic stimulus (8, 27). Different authors have concluded from these enzyme release studies that at concentrations as low as 10^{-6} M colchicine inhibited lysosomal fusion (7, 8, 27), that the drug was effective at high (10^{-4} M) but not at low (10^{-6} M) concentrations (9), or that the drug had no effect at any concentration through 10^{-3} M (35).

Cinephotomicrographic studies have shown that, although the patterns of movement of

both the macrophage and its intracellular granules were altered by treatment with colchicine, no visible inhibition of fusion of endocytic vacuoles with lysosomes was evident (17). Polymorphonuclear leukocytes pretreated with colchicine degraded radiolabeled thyroxine in a manner indistinguishable from controls (36). In addition, Stossel et al. noted that colchicine did not diminish the formation of phagolysosomes by polymorphonuclear leukocytes in their system in which phagolysosomes containing buoyant inert particles are separated from other subcellular particles and unfused lysosomes by density gradient centrifugation (37).

Thus, we were able to confirm and further define the inhibitory effect of colchicine on acid phosphatase induction in the mouse peritoneal macrophage. In addition, data presented in this paper provide further support for previous observations (2) that interiorization of substrate is necessary but not sufficient to promote lysosomal enzyme induction. That an effective lysosomal enzyme inducer must be susceptible to intracellular degradation has been demonstrated (2) and our data imply a further requirement for intact microtubules in acid hydrolase induction by endocytic stimuli.

The site at which colchicine acts to inhibit lysosomal enzyme induction cannot yet be determined. However, recent speculations by Verity (38) and Ehrlich et al. (21) may be relevant. Verity has postulated that invasion of the nucleoplasm by lysosomes is required for lysosomal enzyme induction in certain hormone-sensitive cell lines (38). Such an unusual migration of cytoplasmic particles should require direction and microtubules would be obvious candidates for this role. Ehrlich et al. have demonstrated inhibition of collagen secretion and synthesis by colchicine in embryonic cranial bone cells and suggest that microtubular disruption blocks egress of secretory protein from the Golgi apparatus, with consequent inhibition of synthesis (21). In the macrophage, microtubular disruption could result in a reduced rate at which newly formed primary lysosomes exit to the cytoplasm from the Golgi region.

Summary

The effects of colchicine on lysosomal fusion and lysosomal enzyme induction in the cultivated mouse peritoneal macrophage have been examined. Colchicine (10^{-6} M), but not lumicolchicine, inhibited lysosomal enzyme induction by both phagocytic and pinocytic stimuli. In addition, the drug significantly retarded pinocytic uptake of [3 H]sucrose and transport of the amino acids [3 H]alpha aminoisobutyric acid and L-[3 H]leucine. In contrast, lumicolchicine had no effect on pinocytosis or amino acid transport. Thus, a role for intact microtubules in lysosomal enzyme induction, pinocytosis, and amino acid uptake in these cells is suggested. That colchicine inhibited lysosomal enzyme induction by phagocytic stimuli under conditions in which pinocytosis contributed little to the enzyme rise indicated that inhibition of pinocytosis was unlikely to account for colchicine effects on lysosomal enzyme induction.

Effects of colchicine on degradation of phagocytized and pinocytized substrates were examined to determine if intact microtubules are required for fusion among lysosomes, pinosomes, and phagosomes. Colchicine did not alter the rate of intracellular digestion of radiolabeled bacteria by the cultivated macrophage. Similarly, it had no effect on enzymatic hydrolysis of intracellular [3 H]sucrose

resulting from uptake of exogenous invertase. The finding that colchicine had no effect on the functional consequences of fusion of lysosomes with endosomes suggests that intact microtubules are not required for fusion among these constituents of the vacuolar apparatus.

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