Proc. Natl. Acad. Sci. USA
Vol. 77, No. 1, pp. 480–484, January 1980
Cell Biology

Colchicine inhibits epidermal growth factor degradation in 3T3 cells

(growth control/endocytosis/vinblastine/microtubules/lysosomes)

KENNETH D. BROWN*, MORRIS FRIEDKIN†, AND ENRIQUE ROZENGURT‡

Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London WC 2, England

Contributed by Morris Enton Friedkin, November 1, 1979

ABSTRACT **Colchicine (2 μM) did not affect the initial rate of association of ^{125}I-labeled epidermal growth factor (^{125}I-EGF) to Swiss 3T3 cells but continued incubation (up to 24 hr) led to an increase in cell-associated radioactivity. The effect is also produced by Colcemid, vinblastine, and podophyllotoxin but not by lumicolchicine. Disruption of microtubules with colchicine does not alter the rate of "down regulation" of EGF receptors, suggesting that binding and internalization of the factor proceed unchanged. However, colchicine markedly decreases the rate of appearance of acid-soluble radioactivity from cells either incubated continuously with ^{125}I-EGF for 24 hr or exposed to the radioactive peptide for only 1 or 3 hr. The results indicate that colchicine decreases the rate of degradation of internalized ^{125}I-EGF. Because antitubulin agents enhance the mitogenic effect of EGF, our results suggest that peptide degradation can be dissociated from the long-term biological effect.**

Various purified factors such as epidermal growth factor (EGF), insulin, and fibroblast-derived growth factor stimulate DNA synthesis when added alone or in combination to resting cultures of mouse 3T3 cells (1–3). Recent work from this laboratory (4) has shown that the mitogenic response to these factors is markedly enhanced by colchicine and other agents that cause depolymerization of microtubules. In contrast, lumicolchicine, which does not cause microtubule depolymerization, was without effect.

The interaction of ^{125}I-labeled EGF (^{125}I-EGF) with cultured cells has been extensively investigated (5–9). ^{125}I-EGF binds to specific, saturable receptors on the plasma membrane. Subsequently, the EGF–receptor complex is internalized and the ^{125}I-EGF is degraded in lysosomes with the release of ^{125}I-labeled tyrosine into the medium. Recent work using ferritin-labeled EGF has provided direct morphological evidence in favor of this scheme (10). It was shown that surface bound ferritin-labeled EGF was internalized via endocytic vesicles that subsequently fused with lysosomes.

We have used ^{125}I-EGF to determine whether colchicine alters some stage(s) in the metabolism of EGF by 3T3 cells. We found that the binding and internalization of EGF is unaffected by colchicine but that the rate of degradation of the internalized factor is markedly decreased in the presence of the drug. The possible role of this inhibition of degradation in the enhancement of the stimulation of DNA synthesis is discussed.

MATERIALS AND METHODS

Cell Cultures. Swiss mouse 3T3 and 3T6 cells, propagated as described (1, 2), were subcultured into 35-mm Nunc dishes in Dulbecco–Vogt modified Eagle's medium with 10% (for 3T3) or 0.5% (for 3T6) fetal bovine serum. The cells were fed again after 4 days' growth and incubated for at least a further 4 days before use in the assays.

EGF, ^{125}I-EGF, and Antiserum to EGF. EGF was prepared from mouse submaxillary glands by the method of Savage and Cohen (11). ^{125}I-EGF was prepared as described (9). Rabbits were immunized with unconjugated EGF emulsified in Freund's incomplete adjuvant (Difco) as described (3).

^{125}I-EGF Binding Assay. Cells were washed twice at 37°C with 1–2 ml of binding medium (Dulbecco–Vogt modified Eagle's medium) containing 0.1% crystalline bovine serum albumin, 0.1 μM potassium iodide, and 50 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid adjusted to pH 6.8. The cells were incubated at 37°C with 1 ml of binding medium containing ^{125}I-EGF at the required concentration. After incubation, unbound radioactivity was removed by washing the cells four times with cold (4°C) phosphate-buffered saline (pH 7.4) containing 0.1% albumin and 0.1 μM potassium iodide. The cells were extracted (60 min at 37°C) with 1 ml of 0.5 M NaOH, and cell-associated radioactivity was determined in a gamma counter.

Under these conditions, nonspecific binding, determined as cell-associated radioactivity in the presence of unlabeled EGF at 2 μg/ml, was less than 5% of the total.

Distribution of Cell-Associated ^{125}I-EGF Between the Cell Surface and Cell Interior. This was determined by using a modification of a described method (9). After incubation with ^{125}I-EGF, the cells were washed to remove extracellular radioactivity, and 1 ml of elution medium (binding medium containing 0.5% antiserum to EGF) was added per dish. The dishes were incubated at 37°C. At the indicated times, the elution medium was transferred to test tubes and replaced by 1 ml of fresh elution medium. After a total of 180 min the cells were extracted with NaOH and the remaining cell-associated radioactivity was measured. The acid-insoluble material in the samples of elution medium was precipitated by the addition of trichloroacetic acid (10% final concentration) and incubation at 4°C for 30 min. The precipitate was removed by centrifugation at 4°C. The acid-insoluble ^{125}I activity in the precipitate and the acid-soluble ^{125}I activity in the supernatant were measured. The acid-insoluble ^{125}I-labeled material represents ^{125}I-EGF released from the cell surface. Most of the remaining cell-associated radioactivity was released as acid-soluble ^{125}I-labeled material, indicating that it represented ^{125}I-EGF that had already been internalized at the time of the addition of antiserum. In this procedure the antiserum is required to prevent the internalization of ^{125}I-EGF during the elution (see ref. 9).

Treatment with Colchicine. Cells were pretreated with colchicine by adding 20 μl of a 200 μM solution of the drug directly to the growth medium (2 ml) to give a final concen-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: EGF, epidermal growth factor.

* Present address: ARC Institute of Animal Physiology, Babraham, Cambridge CB2, 4AT England.
† Present Address: Department of Biology, University of California, San Diego, La Jolla, CA 92093.
‡ To whom correspondence should be addressed.

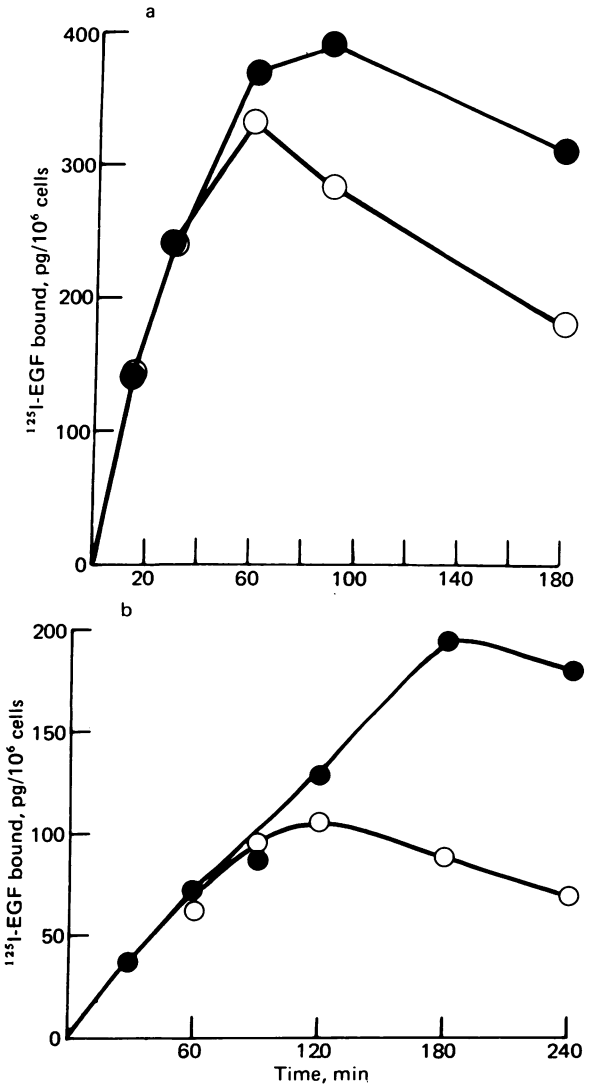

FIG. 1. Time-course of ^{125}I-EGF binding to 3T3 (*a*) and 3T6 (*b*) cells in the absence (○) or presence (●) of colchicine. After a 2-hr preincubation at 37°C with colchicine, the growth medium was replaced by 1 ml of binding medium containing 2 ng of ^{125}I-EGF (128,000 cpm/ng) per ml. The binding medium added to the colchicine-pretreated cells also contained colchicine (2 μM). The dishes were incubated at 37°C for the indicated time and then washed; after this, cell-associated radioactivity was determined. Each point represents the mean (n = 2 or 3) for ^{125}I-EGF binding.

tration of 2 μM. Control cultures received 20 μl of distilled water. After a 2-hr incubation at 37°C, the growth medium was removed from all dishes and replaced by 1 ml of binding medium containing ^{125}I-EGF.

RESULTS

Colchicine (2 μM) did not affect the initial rate of association of ^{125}I-EGF to Swiss 3T3 cells (Fig. 1*a*) or 3T6 cells (Fig. 1*b*). However, continued incubation in the presence of colchicine led to an increase in cell-associated radioactivity in both cell lines. The absence of an effect on initial rates of ^{125}I-EGF binding could be caused by a delay in the expression of colchicine activity. However, this is unlikely because the cells had been pretreated with colchicine for 2 hr prior to the addition of the labeled factor.

Table 1 shows that other antitubulin agents also increased 3T3 cell-associated radioactivity after 4-hr incubation with ^{125}I-EGF. In contrast, lumicolchicine, which does not disrupt

Table 1. Effect of various antitubulin agents on the binding (4 hr) of ^{125}I-EGF to 3T3 cells

Addition*	Conc., μM	Cell-associated ^{125}I-EGF, pg/10^6 cells
None	—	122 ± 4
Colchicine†	0.2	290 ± 2
	2	313 ± 1
Vinblastine	2	325 ± 4
Colcemid	2	272 ± 8
Podophyllotoxin	2	262 ± 3
Lumicolchicine	0.2	118 ± 2
	2	123 ± 1

Results are shown as mean ± SEM for triplicate binding determinations.

* Cells were pretreated for 2 hr with the drug at the indicated concentration. Cell-associated radioactivity was measured after a 4-hr incubation at 37°C with binding medium containing 1 ng of ^{125}I-EGF (102,000 cpm/ng) per ml and the antitubulin agent.

† In a separate similar experiment, cells were exposed to a range of colchicine concentrations (1 nM–1 μM). A maximal increase in cell-associated radioactivity was found at concentrations higher than 0.1 μM; 0.01 μM was without effect.

microtubules, did not increase cell-associated ^{125}I-EGF above the control value.

The effect of colchicine on the association of ^{125}I-EGF to 3T3 cells was further investigated during a 24-hr incubation with labeled factor (Fig. 2*a*). In both treated and untreated cells, cell-associated radioactivity was maximal at 1 hr and subsequently declined on continued incubation. However, the rate of this decrease was markedly reduced in the colchicine-treated cells. Thus, at all times after 1 hr, colchicine produced an increase in cell-associated radioactivity.

In the same experiment, the degradation of ^{125}I-EGF by the cells was determined by measuring the release of acid-soluble ^{125}I-labeled material into the medium (Fig. 2*b*). Acid-soluble radioactivity began to accumulate in the medium of untreated cells after a 1-hr lag; it increased rapidly between 1 and 3 hr and more slowly thereafter. This result is similar to that reported (9) for BALB 3T3 cells. In the presence of colchicine, the lag before acid-soluble ^{125}I-labeled material appeared in the medium was increased to 2 hr and the initial rapid rate of accumulation was reduced. Thus, during a 4-hr incubation, the control cells degraded 218 pg of ^{125}I-EGF per dish, whereas the colchicine-treated cells degraded only 99 pg per dish. The control cultures showed a higher level of ^{125}I-EGF degraded until 24 hr, at which time treated and untreated cultures had both degraded approximately 550 pg of ^{125}I-EGF per dish. Therefore, 55% of the 1 ng of ^{125}I-EGF added per dish was degraded during the course of the experiment.

The increase in cell-associated ^{125}I-EGF in the presence of colchicine could be due to: (*i*) an increase in the binding of ^{125}I-EGF to cell surface receptors, although this is unlikely because the initial rate of ^{125}I-EGF association was not altered by colchicine (Fig. 1); (*ii*) a decrease in the rate of internalization of the EGF–receptor complex, which would decrease the intracellular ^{125}I-EGF available for degradation; (*iii*) an unaltered rate of internalization of the EGF–receptor complex but a decrease in the rate of degradation of internalized ^{125}I-EGF.

The results in Fig. 3 demonstrate that colchicine did not affect the rate of down regulation of EGF receptors induced by unlabeled EGF at either 1 or 10 ng/ml. This finding suggests that colchicine does not affect the binding and internalization of EGF. Therefore, the increase in cell-associated radioactivity in the presence of colchicine is most likely due to a reduction in the rate of intracellular degradation of ^{125}I-EGF.

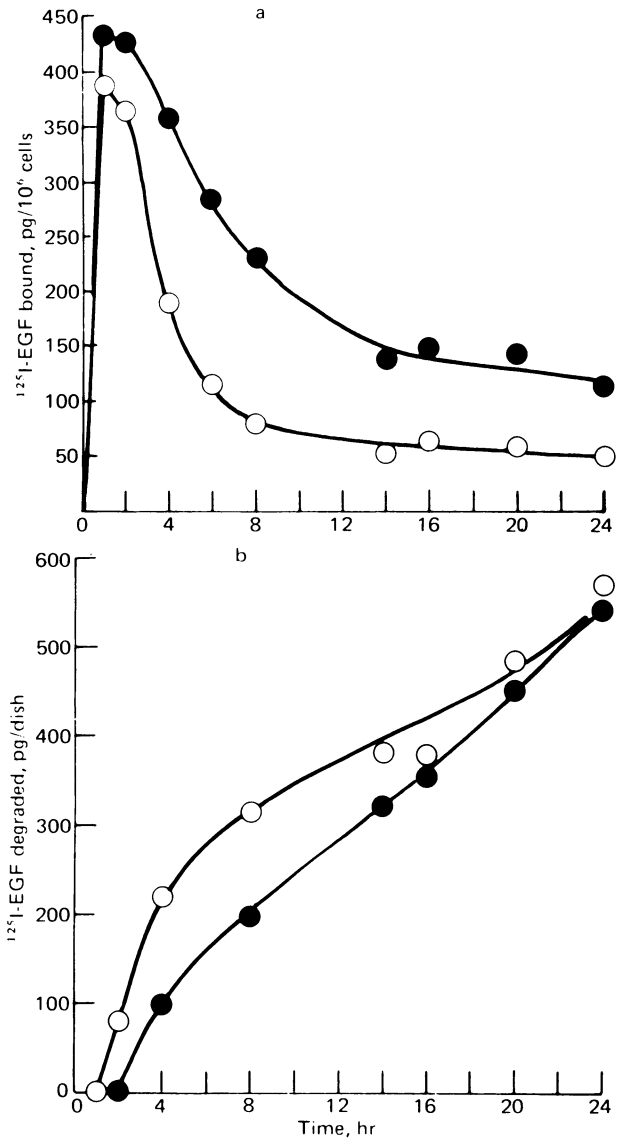

FIG. 2. Effect of colchicine on the binding (*a*) and degradation (*b*) of ^{125}I-EGF by 3T3 cells. Pretreatment with colchicine and binding with ^{125}I-EGF were carried out as described in the legend to Fig. 1 except that the concentration of ^{125}I-EGF in the binding medium was 1 ng/ml (73,000 cpm/ng). At the indicated times, the binding medium was removed into test tubes and cell-associated radioactivity was determined. The acid-insoluble material in the samples of binding medium was precipitated by the addition of trichloroacetic acid (10% final concentration) and incubation for 30 min at 4°C. The precipitate was removed by centrifugation at 4°C. The acid-soluble ^{125}I activity in a 1-ml sample of the supernatant was measured. All values were corrected for acid-soluble ^{125}I activity present in the ^{125}I-EGF preparation. This background value did not increase after a 24-hr incubation of binding medium in the absence of cells. Each point represents the mean obtained from two dishes of cells, ○, Control; ● 2 μM colchicine.

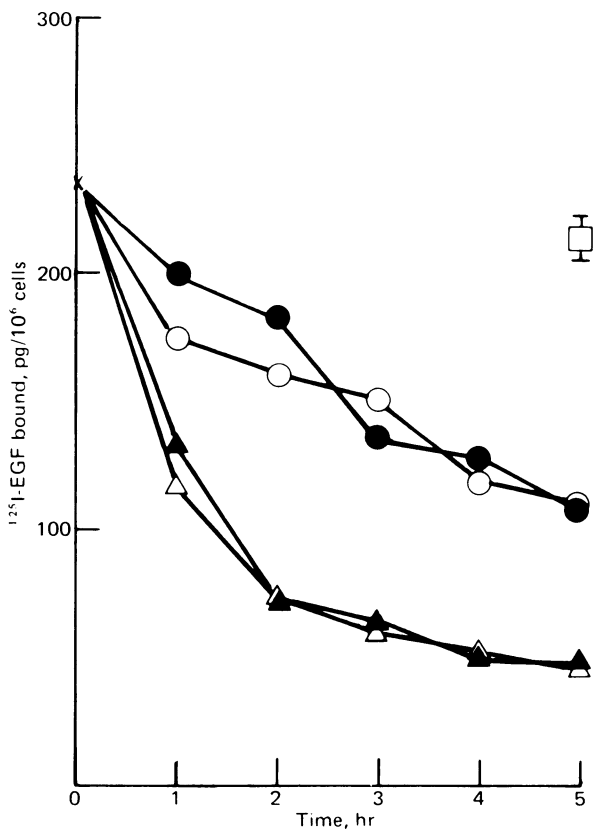

FIG. 3. Down regulation of EGF receptors by EGF in the presence (closed symbols) or absence (open symbols) of colchicine. After 2-hr pretreatment with colchicine, unlabeled EGF was added at a final concentration of 1 ng/ml (circles) or 10 ng/ml (triangles). The cells were incubated at 37°C. At the indicated times, the cells were washed twice with binding medium, and 1 ml of binding medium containing ^{125}I-EGF (1.2 ng/ml; 83,000 cpm/ng) was added per dish. The cells were incubated for 60 min at 37°C, and cell-associated radioactivity was measured. Each point represents the mean value from two dishes. □, Effect of a 5-hr incubation with binding medium without EGF on ^{125}I-EGF binding.

If this interpretation is correct, the distribution of ^{125}I-EGF between the cell surface and the intracellular compartment after a 1-hr exposure to ^{125}I-EGF should be unaltered by colchicine. However, the rate at which internalized ^{125}I-EGF is subsequently released as acid-soluble ^{125}I-labeled material should be decreased in colchicine-treated cells. This was examined by incubating cells for 1 hr with ^{125}I-EGF, removing extracellular ^{125}I-EGF, and measuring acid-insoluble and acid-soluble ^{125}I-labeled material released by the cells at various times during a subsequent 3-hr incubation with medium containing antiserum to EGF. The total amount of cell-associated ^{125}I-EGF was similar in the presence or absence of colchicine (time 0, Fig. 4*a*). The amount of ^{125}I-EGF bound at the cell surface [released as acid-insoluble ^{125}I-labeled material (Fig. 4*b*)] and the amount of ^{125}I-labeled material released from the intracellular compartment (acid-soluble, Fig. 4*c*) were similar in treated and untreated cultures at the end of the experiment. However, the *rate* at which the acid-soluble ^{125}I-labeled material was released from the cells was significantly decreased by colchicine (Fig. 4*c*). This decreased rate of release of acid-soluble material accounted for the decrease in the *overall* rate of release of ^{125}I-labeled material in the presence of colchicine (Fig. 4*a*).

For comparison, the effects of NH_4Cl, which is known to decrease ^{125}I-EGF degradation, presumably by inhibition of lysosomal function (6), also are shown in Fig. 4. The results show that, like colchicine, NH_4Cl did not greatly affect the binding and internalization of ^{125}I-EGF by 3T3 cells. However, the degradation of intracellular ^{125}I-EGF was decreased by NH_4Cl to such an extent that, at the end of the experiment, 48% of the initial cell-associated radioactivity remained cell-associated (Fig. 4*a*).

An experiment similar to that shown in Fig. 4 but with a 3-hr exposure to ^{125}I-EGF demonstrated that, as expected, total cell-associated radioactivity was increased in the presence of

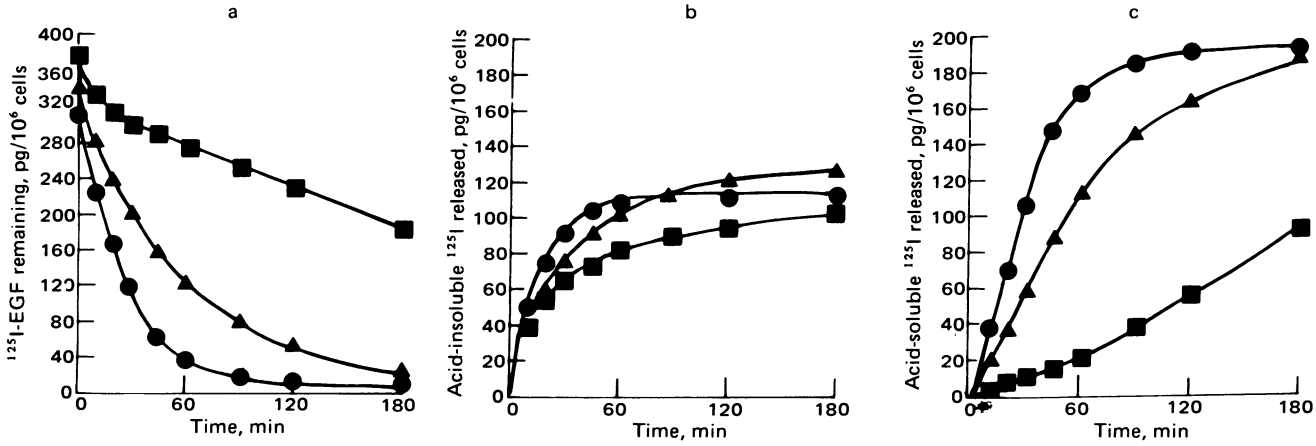

FIG. 4. Effect of colchicine and NH_4Cl on the release of total (*a*), acid-insoluble (*b*), and acid-soluble (*c*) ^{125}I-labeled material from 3T3 cells previously exposed to ^{125}I-EGF. Pretreatment with colchicine or NH_4Cl and binding of ^{125}I-EGF (2 ng/ml; 81,000 cpm/ng) for 1 hr at 37°C were as described in the legend to Fig. 1. The subsequent elution of cell-associated ^{125}I-labeled material was followed. ●, Control; ▲, 2 μM colchicine; ■, 10 mM NH_4Cl.

colchicine. During subsequent incubation after the removal of extracellular ^{125}I-EGF, the amount of ^{125}I-EGF released from the cell surface was similar in treated and untreated cultures. The additional cell-associated radioactivity was entirely due to an accumulation of intracellular ^{125}I-EGF as indicated by an increase in the amount of acid-soluble material released (results not shown).

DISCUSSION

The pathway of EGF metabolism by cultured cells—binding, internalization, and degradation—is now well established. However, the question of which stage(s) is involved in generating a mitogenic signal(s) remains unknown. There are two major approaches to answering this question. The genetic approach involves isolating cell mutants with defects at each stage of EGF metabolism in order to determine which events are necessary for the stimulation of DNA synthesis. To date, the only progress along these lines has been the isolation of receptor-negative mutants of 3T3 cells (12). The pharmacological approach involves using drugs to alter the various stages in EGF metabolism and attempting to correlate the observed effect with changes in the biological response to the growth factor.

We have previously demonstrated that colchicine enhances the response of 3T3 cells to EGF (4). Thus, our results demonstrate that a decreased rate of growth factor degradation not only does not prevent biological activity but actually is associated with an enhancement of the mitogenic effect of EGF. It has been hypothesized that EGF degradation might be an essential step in mediating the mitogenic response (13). Our results show that factor degradation and long-term biological activity (stimulation of DNA synthesis) can be dissociated in colchicine-treated cells.

It has recently been shown that colchicine inhibits the degradation of low density lipoprotein by human skin fibroblasts (14) and the degradation of asialo glycoproteins by isolated rat hepatocytes (15). In contrast to its effects on EGF metabolism, colchicine also inhibits the endocytic internalization of receptor-bound low density lipoprotein and decreases the binding capacity of the plasma membrane for asialo glycoproteins. However, these effects of colchicine on the uptake and degradation of macromolecules by cultured cells are not universal. The drug has no effect on ^{125}I-labeled bovine serum albumin degradation by human skin fibroblasts (14), and the binding kinetics of ^{125}I-labeled diphtheria toxin to BSC-1 cells at 37°C is unaffected by colchicine (16).

The mechanism by which colchicine inhibits the degradation of EGF is not known. The inhibition may be due to a direct effect on lysosomal function such as presumably occurs with NH_4Cl and chloroquine (6). Alternatively, colchicine may prevent the fusion of the internalized endocytic vesicles (10) with lysosomes. The latter possibility is supported by the evidence of Ostlund *et al.* (14) who used immunofluorescence techniques to demonstrate that, in the presence of colchicine, lysosomes tended to accumulate in the perinuclear region, whereas endocytic vesicles containing low density lipoprotein were found at the cell periphery. Other workers have reported that colchicine inhibits the saltatory movement of lysosomes (17) and endosome-lysosome fusion (18), indicating that intact microtubules are required for these processes.

The effects of colchicine on EGF degradation and mitogenic activity (4), the recent morphological data on EGF metabolism (10), and the fact that colchicine may prevent the fusion of endocytic vesicles with lysosomes (14, 18) have prompted the following speculation. We propose that EGF, bound to its receptor in endocytic vesicles, may remain coupled to an "effector system" and be capable of mitogenic signal generation. In this way, the mechanisms of action of surface-bound and internalized EGF may not be essentially different. In this scheme, colchicine would enhance EGF activity by inhibiting the fusion of EGF-containing endocytic vesicles with lysosomes.

We thank Patricia Pettican for expert technical help. M.F. was supported by an American Cancer Society Eleanor Roosevelt International Cancer Fellowship and in part by Grant CA11449 from the National Cancer Institute.

1. Dicker, P. & Rozengurt, E. (1978) *Nature (London)* **276**, 723–726.
2. Bourne, H. & Rozengurt, E. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 4555–4559.
3. Brown, K. D. & Holley, R. W. (1979) *J. Cell. Physiol.* **100**, 139–146.
4. Friedkin, M., Legg, A. & Rozengurt, E. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3909–3912.
5. Carpenter, G., Lembach, K. J., Morrison, M. M. & Cohen, S. (1975) *J. Biol. Chem.* **250**, 4297–4304.
6. Carpenter, G. & Cohen, S. (1976) *J. Cell Biol.* **71**, 159–171.
7. Vlodavsky, I., Brown, K. D. & Gospodarowicz, D. (1978) *J. Biol. Chem.* **253**, 3744–3750.
8. Ahronov, A., Pruss, R. M. & Herschman, H. R. (1978) *J. Biol. Chem.* **253**, 3970–3977.
9. Brown, K. D., Yeh, Y.-C. & Holley, R. W. (1979) *J. Cell. Physiol.* **100**, 227–238.
10. Haigler, H. T., McKanna, J. A. & Cohen, S. (1979) *J. Cell Biol.* **81**, 382–395.

11. Savage, C. R. & Cohen, S. (1972) *J. Biol. Chem.* **247,** 7609–7611.
12. Pruss, R. M. & Herschman, H. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3918–3921.
13. Das, M. & Fox, C. F. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2644–2648.
14. Ostlund, R. E., Pfleger, B. & Schonfeld, G. (1979) *J. Clin. Invest.* **63,** 75–84.
15. Kolset, S. O., Tolleshaug, H. & Berg, T. (1979) *Exp. Cell Res.* **122,** 159–167.
16. Middlebrook, J. L., Dorland, R. B. & Leppla, S. H. (1979) *Exp. Cell Res.* **121,** 95–101.
17. Freed, J. J. & Lebowitz, M. M. (1970) *J. Cell Biol.* **45,** 334–354.
18. Malawista, S. E. & Bodel, P. T. (1967) *J. Clin. Invest.* **46,** 786–796.