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THE SYNTHESIS OF THE BRAIN SPECIFIC S100 PROTEIN IN COLCEMID RESISTANT MUTANTS OF RAT GLIAL CELLS

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We have isolated two colcemid-resistant mutant sublines, CM^R (7A) and CM^R (7B), from rat glial cells, C6, using multiple consecutive selections with increasing concentrations of colcemid. The mutant sublines show a decreased uptake of [^{3}H]colchicine but have no apparent defect in the cytoplasmic binding of the drug. The synthesis of the brain-specific S100 protein is less sensitive to colcemid inhibition in the mutant cell lines than in parental C6 cells, suggesting that colcemid must enter the cell to inhibit S100 protein synthesis.

Treatment of rat glial cells, C6, with antimicrotubular drugs inhibits the production of a small number of specific proteins which include the brain-specific S100 protein (1-4). The inhibition of synthesis of S100 protein by colchicine is paralleled by a decrease in the activity of S100 protein messenger RNA (mRNA) (5). The effect of antimicrotubular drugs on S100 protein synthesis is presumably secondary to a disruption of microtubules since it can be elicited by a wide range of antimicrotubular drugs acting on intact cells but not on cellular homogenenates (1,4). Lumicolchicine an inactive analog of colchicine has no effect on S100 protein synthesis (1). There are no known mechanisms to explain how a generalized perturbation of the cell caused by a disruption of microtubules can exert a specific effect on individual proteins. In order to study further the action of antimicrotubular drugs on S100 protein synthesis, we have isolated two colcemid-resistant (CM^R) mutant cell lines of C6 cells. The basis of resistance of these cell lines to colcemid is a decreased uptake of the drug. The synthesis of S100 protein in these cell lines is partially resistant to inhibition by colcemid. These results suggest that antimicrotubular drugs must enter the cell in order to exert their effect on S100 protein synthesis.

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MATERIALS AND METHODS

1. Materials

Colchicine, puromycin dihydrochloride, ethylmethanesulfonate (EMS) and Tween 80 (polyoxyethylene sorbitan monooleate) were from Sigma (St. Louis, Mi). Colcemid was from Gibco (Burlington, Ontario). [^{35}S]methionine (< 500 Ci/mmol) and methoxy-[^{3}H]colchicine (23 Ci/mmol) were from New England Nuclear (Lachine, P.Q.). L-[4,5-^{3}H]leucine (60 Ci/mmol) was from Amersham (Oakville, Ontario).

2. Growth of cells, measurement of the relative synthesis of S100 protein

The growth of C6 cells, treatments with antimicrotubular drugs, incorporation of [^{35}S]methionine into cells and determination of the relative synthesis of S100 protein have been described before (1,4). Colony formation was determined by plating 10^2-10^3 cells in 25 cm^2 tissue culture dishes (Falcon Plastics, Oxnard, Ca) and scoring for macroscopically visible colonies after incubation at 37°C for 10 days.

3. Selection of C6 cell mutant sublines resistant to colcemid

Mutant sublines were isolated in multiple consecutive selections using increasing concentrations of colcemid after mutagenesis with EMS (6). Approximately 1-2 x 10^7 C6 cells in the logarithmic phase of growth were treated with EMS (100 ug/ml) for 20 hours at 37°C in alpha MEM medium (Gibco) supplemented with 4% fetal calf serum (FCS). The cells were grown for 3 days in the absence of the mutagen to allow expression of mutations before addition of selective drugs. Selection was performed by addition of colcemid .015-.075 ug/ml and Tween 80 (25 ug/ml) to the growing cultures. After two weeks the surviving cells were plated under colony forming conditions in the presence of colcemid. The surviving colonies were picked with a Pasteur pipette and grown up in mass culture. The concentrations of EMS (100 ug/ml) and Tween 80 (25 ug/ml) chosen for mutagenesis and selection respectively inhibited plating efficiencies of C6 cells by approximately 60%. The mutant sublines CM^R (7A) and CM^R (7B) were selected for growth in the presence of colcemid (.075 ug/ml) and Tween 80 (25 ug/ml). In these selective conditions parental C6 cells do not survive and plate at an efficiency of $<10^{-6}$.

4. Biochemical assays

The assays of [^{3}H]colchicine uptake and binding were adapted from those published in the literature (6-8). For [^{3}H]colchicine uptake cells in the logarithmic phase of growth were harvested from monolayers with Viokase (Gibco); 5 x 10^6 cells were suspended in 1 ml of Hanks balanced salt solution (HBSS, Gibco) and incubated at 37°C with 2 uCi of [^{3}H]colchicine (2.5 x 10^{-7} M). At various times aliquots of 100 ul (5 x 10^5 cells) were collected by centrifugation at 4°C and washed with 0.5 ml of phosphate buffered saline (PBS, 140 mM NaCl, 3 mM KCl, 0.15 mM KH_2PO_4, 8 mM $Na_2HPO_4.7H_2O$ pH 7.1), containing 5 x 10^{-4}M colchicine. The cell pellet was suspended in 10 ml of Aquasol (New England Nuclear) and counted in a liquid scintillation spectrometer.

For [^{3}H]colchicine binding, approximately 3 x 10^7 logarithmically growing cells were harvested as described above, suspended in an equal volume (0.5 ml) of 200 mM MES (2-(N-morpholino) ethanesulfonic acid) buffer, pH 6.8, containing 2 mM $MgCl_2$, 2 mM EGTA, 4 mM DTT, broken by sonication and centrifuged at 25,000 x g for 15 min. The supernatants served as a source of cytoplasmic extract for binding assays. Binding was performed by incubating 50 ul of the cytoplasmic lysate with 50 ul of [^{3}H]colchicine, 2 uCi, for 2 hours at 37°C. The concentrations of colchicine were adjusted to a range of 7.5 x 10^{-7} - 6 x 10^{-6} M. To separate bound from free colchicine 20 ul of the reaction mixture was chromatographed directly on a 5 ml Sephadex G25 (Pharmacia, Dorval, P.Q.) column equilibrated with the buffer used for sonication. Fractions of 0.5 ml were collected, mixed with 5.0 ml of Aquasol and counted in a liquid scintillation spectrometer. [^{3}H]colchicine bound to the cytoplasm was excluded in the first four fractions.

To assess resistance to puromycin, monolayers of cells, 10^6 cells/dish, were incubated with 15 uCi/ml of [^{3}H]leucine in leucine-free alpha MEM at 37°C for 60 min

in the presence or absence of puromycin. Incorporation of [^{3}H]leucine into cellular proteins was determined as described previously (1,4).

RESULTS

1. Characterization of CM^R cells

Since colcemid is a close analog of colchicine and competes with colchicine for binding to tubulin (7) we used [^{3}H]colchicine to characterize the CM^R mutant cell lines. Both CM^R (7A) and CM^R (7B) cell lines show a decreased uptake of [^{3}H]colchicine in comparison with parental C6 cells, with CM^R (7B) cells having a greater defect in the uptake of the drug (Figure 1). This decreased uptake could be due to an interference with the permeability of the drug across the cell membrane or to a defective binding of the drug to intracellular microtubules. In order to test

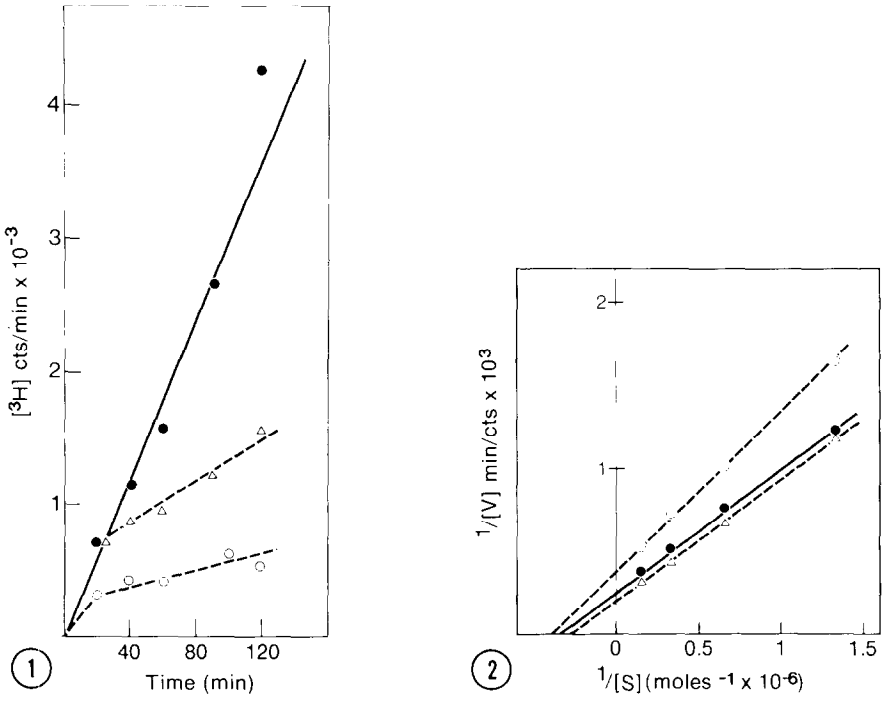

Fig. 1. Uptake of [^{3}H]colchicine
$5x10^6$ cells were incubated with [^{3}H]colchicine as described in Methods. At various times aliquots of $5x10^5$ cells were washed and counted.
● C6 cells Δ CM^R (7A) cells o CM^R (7B) cells

Fig. 2. Binding of [^{3}H]colchicine to cytoplasmic extracts
Aliquots of cytoplasmic lysates were incubated with [^{3}H]colchicine, 7.5×10^{-7} - 6×10^{-6} M for 2 hours at 37°C. Colchicine bound to cytoplasmic proteins was separated from unbound colchicine by gel filtration and quantitated by counting in a liquid scintillation spectrometer. [S] is concentration of colchicine in M. [V] is cts/min in bound fraction.
● C6 cells Δ CM^R (7A) cells o CM^R (7B) cells

for the latter possibility cytoplasmic lysates of CM^R cells were incubated with [^{3}H]colchicine for two hours at concentrations of colchicine ranging from 7.5×10^{-7} to 6×10^{-6} M. During this period the binding of the drug to cytoplasmic extracts is linear with time (7). The amount of [^{3}H]colchicine bound after two hours was taken as the initial velocity of the reaction and the data analyzed by a Lineweaver-Burk plot. The intercept on the abscissa represents -1/Km of the reaction. The results show that the Km is similar for the reactions with cytoplasmic extracts from parental C6 cells, CM^R (7A) cells and CM^R (7B) cells (Figure 2). This suggests that the affinity of the microtubules for colchicine is similar in the parental and the mutant cells.

In order to test for a more generalized permeability defect which could account for a decreased uptake of [^{3}H]colchicine by the mutant cell lines, CM^R (7A) and CM^R (7B) cells were tested for a cross-resistance to puromycin (9). CM^R (7A) cells were as sensitive to puromycin as the parental C6 cells, whereas CM^R (7B) cells were resistant to the drug (Figure 3).

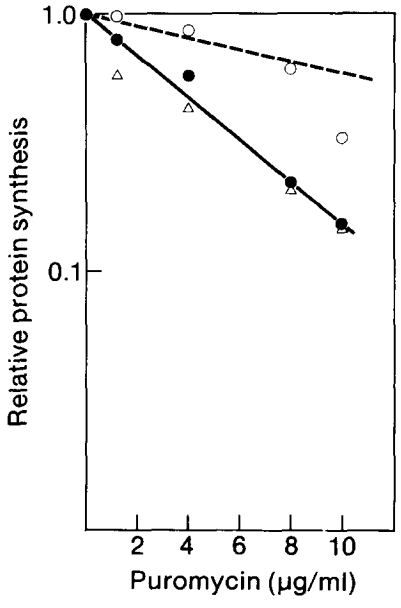

Fig. 3. Inhibition of protein synthesis by puromycin
Monolayers were incubated with [^{3}H]leucine in the presence or absence of puromycin for 60 min at 37°C. Incorporation of [^{3}H]leucine into proteins was expressed relative to cells incubated without puromycin •C6 cells
Δ CM^R (7A) cells o CM^R (7B) cells

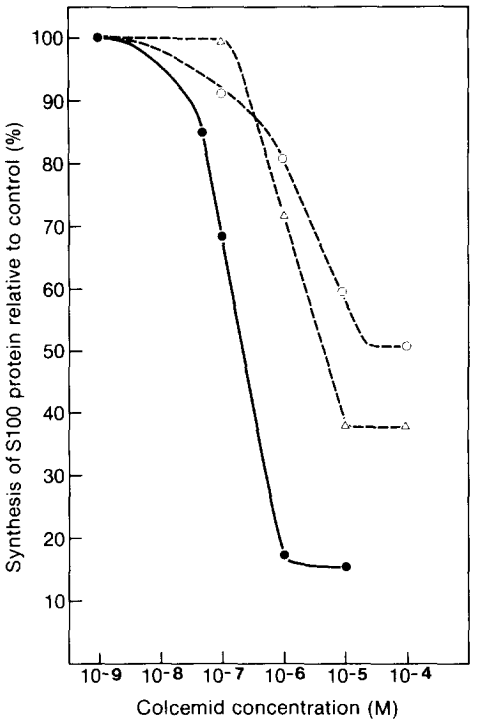

Fig. 4. Dose response curve for inhibition of S100 protein by colcemid
Confluent monolayers were treated with colcemid at indicated concentrations for 19 hours at 37°C. [^{35}S]methionine 8.5 uCi/ml was added for the last 3 hours of treatment. The cells were harvested and the relative synthesis of S100 protein determined as previously described. Synthesis of S100 protein relative to control = (relative synthesis of S100 protein in colcemid-treated cultures/relative synthesis of S100 protein in cultures without colcemid) x 100. (1)
● C6 cells ▲ CM^R (7A) cells o CM^R (7B) cells

2. S100 protein synthesis in CM^R mutant cell lines

The relative synthesis of S100 protein was inhibited in confluent cultures of C6 cells by colcemid as previously reported (1). In both CM^R (7A) and CM^R (7B) cells the synthesis of S100 protein was more resistant to inhibition by colcemid than in parental C6 cells (Figure 4).

DISCUSSION

Cellular microtubules are involved in a number of essential cellular functions including formation of the spindle structure during mitosis and maintaining the cytoskeleton during interphase. Mutant Chinese hamster ovary (CHO) cell lines resistant to the action of antimicrotubular drugs have been previously characterized in other laboratories (6-8,10). These mutant cell lines fall into two general classes. The first is composed of permeability mutants which have a defect in the

penetration of a number of lipophilic drugs including antimicrotubular drugs and puromycin across the cell membrane (7,9,11). The second is composed of mutants with altered tubulin which is manifested either by a decreased binding affinity of tubulin for antimicrotubular drugs (6,8) or by changes in the electrophoretic mobility of the α or β tubulin subunits (8,10). Of the two mutant sublines of C6 cells described here, CM^R (7B) is apparently a permeability mutant because of its cross-resistance to puromycin (Figure 3). Although our selection was performed in the presence of the detergent Tween 80 in order to promote the entry of colcemid into cells, the amount of Tween 80 used, (25 ug/ml), was lower than that used by other authors, (100 ug/ml), to avoid selection of permeability mutants of CHO cells (6). This may explain our selection of a permeability mutant in the presence of the detergent. However the possibility that CM^R (7B) cells have associated defects in transport rather than in permeability of certain drugs across the cell membrane cannot be excluded. The basis of resistance of CM^R (7A) cells is less clear. Since this cell line is not cross-resistant to puromycin (Figure 3) it may have a selective permeability defect to some drugs which include colcemid and colchicine. It may also have a defect which interferes with the binding of colcemid and colchicine to microtubules, although this defect is not apparent in the cytoplasmic binding assay (Figure 2). In this respect it should be noted that CHO mutants with altered β tubulin subunits but with no altered binding of [^{3}H]-colchicine in cell-free extracts have been reported (10). Since multiple consecutive selections can lead to an accumulation of several independent mutations each contributing to resistance to colcemid, it is possible that CM^R (7A) and CM^R (7B) cells have a complex mutant phenotype (8). It is noteworthy that while the two mutant cell lines were picked as individual clones from the same population, their phenotypes are clearly different.

The synthesis of S100 protein was less sensitive to inhibition by colcemid in both CM^R (7A) and CM^R (7B) cells than in parental C6 cells (Figure 4). This indicates that entry of the drug into the cells is a prerequisite for inhibition of S100 protein synthesis. This observation supports the hypothesis that depolymerization of microtubules can affect the synthesis of specific proteins (1,4). It is not known if depolymerization of microtubules triggers one or more

intermediate reactions which mediate this effect. Nonetheless it is remarkable that only a small number of proteins are specifically affected in C6 cells (1-4). In the case of S100 protein our previous observations suggest that the inhibition of synthesis following colchicine treatment occurs at the level of production of S100 protein mRNA (5). In cultured fibroblasts colchicine treatment was shown to decrease the synthesis of tubulin and the activity of tubulin mRNA. It was suggested that depolymerization of microtubules increased the pool of free tubulin subunits which inhibited one of the steps in the production of tubulin mRNA (12). A different mechanism must underlie the inhibition of synthesis of non-tubulin proteins, unless the production of their mRNA's is linked with the production of tubulin mRNA. The availability of different colcemid-resistant mutant clones of C6 cells carrying different molecular changes should help to elucidate the mechanism by which depolymerization of microtubules affects the synthesis of specific proteins.

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