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Regulation of Acetylcholinesterase in Neuroblastoma Cells

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Abstract. The specific activity of mouse neuroblastoma acetylcholinesterase (EC 3.1.1.7) increased 25-fold when the rate of cell division was restricted. The results show that acetylcholinesterase activity is regulated in neuroblastoma cells and that the regulatory mechanism is inversely related to the rate of cell division. Under the same conditions the specific activity of catechol-*O*-methyl transferase (EC 2.1.1.6) did not change significantly.

Mouse neuroblastoma C-1300 has been transplanted from animal to animal approximately 1000 times over a period of 30 years; nevertheless, cells, after 5000–8000 cell generations, continue to follow a program of neuron differentiation. Cloned cells contain acetylcholinesterase (AChE), choline acetyltransferase (EC 2.3.1.6), and enzymes for norepinephrine synthesis.[1,2] Cells also are capable of extending processes, several millimeters in length, that contain microtubules,[3,5] neurofilaments, and dense core vesicles[2] and possess membranes capable of generating action potentials in response to electrical stimulation[4,6–8] or acetylcholine.

Burkhalter *et al.*[9] and Goodwin and Sizer[10] have shown that the acetylcholinesterase activity of cultured chick embryo intestine and skeletal muscle can be regulated. In this report the activities of mouse neuroblastoma acetylcholinesterase and catechol-*O*-methyl transferase were investigated.[11] We wish to report that neuroblastoma acetylcholinesterase activity is regulated and that the regulatory process is coupled to the rate of cell division.

Materials and Methods. Cells: Experiments were performed with mouse neuroblastoma C-1300, clone N-18,[5] passage 43-60. Cells were grown in Dulbecco's modification of Eagle's medium supplemented with: 50 units of penicillin G and 10 μg of streptomycin sulfate/ml, 10% fetal calf serum, and 10% horse serum (except where noted) in 10% CO_2–90% air. Cells were kept in logarithmic growth in monolayer culture (i.e., cells grown attached to the surface of a Petri dish) for 7–10 generations before each experiment. For experiments, cells were grown in 150-mm Petri dishes (Falcon Co.) containing 20 ml of growth medium. The medium was changed every other day. Cells in logarithmic phase growth were dissociated by incubation without Ca^{2+} or Mg^{2+} for 10–15 min in saline D_1,[12] supplemented with 5.6 mM glucose and 59 mM sucrose (modified D_1). Cells in stationary phase growth were firmly attached to the growth surface, and their subculture required an additional incubation with 0.05% "trypsin" (Difco 1:250) in modified D_1 for 10–15 min at 37°C.

Homogenates: The medium from each dish was transferred to a centrifuge tube and the cell monolayer was washed 3 times with modified D_1. Washes and growth medium were

pooled and centrifuged (250 × *g*, 10 min, 3°C); the cell pellet was suspended in modified D_1, then washed twice more in the same manner. Dishes were drained, and cell monolayers recovered by scraping. Dishes were then washed twice with small amounts of a solution containing 50 mM potassium phosphate buffer, pH 6.8, and 1 mM EDTA. The washes, scraped cells, and cell pellet were combined and sonicated. Homogenates were stored in small portions at −196°C. No effect of storage on AChE or catechol-*O*-methyl transferase activities was detected.

Assays: Cell concentrations were determined in duplicate with a hemocytometer, cell viability was determined by the nigrosin method,[13] and protein was determined by a modification of the method of Lowry *et al.*[14]

Catechol-*O*-methyl transferase activity was determined by a modification of the method of Nikodejevic, Senoh, Daly, and Creveling.[15] Each reaction contained the following, in a final volume of 0.05 ml: 5 mM potassium phosphate buffer, pH 6.8; 0.8 mM EDTA; 5 mM $MgCl_2$; 1 mM dihydroxybenzoic acid; 1.1 mM [^{14}C]methyl-*S*-adenosyl-L-methionine iodide (4.6 μCi/μmol); and homogenate protein. Reactions were incubated at 37°C for 20 min. The rate of reaction was proportional to enzyme concentrations. Each reaction mixture was acidified and then extracted with toluene.

AChE was measured by a modification of the method of Reed *et al.*,[17] to be described in detail elsewhere (S. Wilson, *et al.*, in preparation). In brief, 1-[^{14}C]acetylcholine is incubated with homogenate; then 1-[^{14}C]acetate is separated from the substrate and counted. Each reaction contained the following components, in a final volume of 0.05 ml, except where noted: 0.05 M potassium phosphate buffer, pH 6.8; 0.2 M NaCl; 1 mM EDTA; 0.5% Triton X-100 (Packard Instrument Co.); 3.3 mM 1-[^{14}C]acetylcholine iodide (0.39 μCi/μmol); and 0–0.05 mg homogenate protein. Reactions were incubated for 10 min at 37°C except where noted, and terminated by the addition of 1.5 ml of a solution, at 3°C, containing 2 μM 1,5-bis-(4-allyldimethylammoniumphenyl) pentane-1,3-dibromide (BW284C51, Burroughs-Wellcome Co.), an inhibitor of AChE.[16] In all cases, the rate of reaction was proportional to homogenate concentration. The diluted reaction mixture, and a subsequent 1.5 ml wash containing the AChE inhibitor, were passed through a disposable 0.5 × 5 cm column of BioRad AG50X8 (Na^+-form, 100–200 mesh) washed with water. The eluates were collected in a scintillation vial, 10 ml scintillation fluid (1000 g Triton X-100–2000 ml toluene–165 ml Liquifluor [New England Nuclear]) was added and the radioactivity was determined. Most values reported represent the average of triplicate homogenates; each homogenate was assayed at four concentrations. The reproducibility of AChE and catechol-*O*-methyl transferase specific activity determinations is ±15%.

One unit of AChE or catechol-*O*-methyl transferase activity is defined as 1.0 nmol [^{14}C]-product formed per minute. Specific activity of the enzymes is expressed as units of enzyme per mg protein. Enzyme activity per dish corresponds to units of enzyme per dish.

Results. Enzyme activity as a function of cell division: The relation between AChE and catechol-*O*-methyl transferase activities and the rate of multiplication of neuroblastoma cells is illustrated in Fig. 1. Each Petri dish was inoculated with 2 × 10^6 cells. After a slight lag, cells multiplied rapidly with a generation time of 24 hr. The maximum cell concentration (28 × 10^6 cells) was attained on approximately the 6th day of incubation. The specific activity of AChE did not change appreciably during the period of rapid cell division. However, during the stationary phase of growth, the 6th through the 17th day of incubation, the specific activity of AChE increased 25-fold.

To investigate the effect of logarithmic growth upon AChE specific activity, stationary phase cells were subcultured at lower cell concentrations on the 13th day of incubation and then maintained in logarithmic growth. A 50% decrease

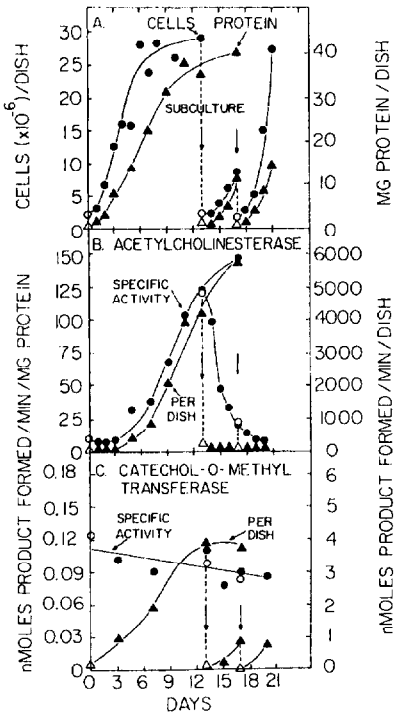

FIG. 1. Neuroblastoma cells in logarithmic growth were subcultured without trypsin. On the 13th and 17th days of incubation, some of the cells were dissociated with trypsin (indicated by the vertical dotted lines). Closed and open symbols represent the values obtained before and after subculture, respectively. In *panel A*, cells/dish (●) and mg protein/dish (▲) are shown. More than 85% of the cells were viable throughout the experiment. In *panel B*, AChE specific activity (●) and AChE activity/dish (▲) are shown. In *panel C*, catechol-*O*-methyl transferase specific activity (●) and activity/dish (▲) are presented.

in AChE specific activity was observed within 40 hr; after 6 days the specific activity returned to the basal level.

The amount of protein per average cell doubled, from 0.6 to 1.2 ng after cells shifted from logarithmic to stationary growth. Over the same period, AChE activity per dish increased greatly, resulting in a 50-fold rise in AChE/cell. When stationary phase cells were shifted to logarithmic growth, corresponding decreases in protein and AChE per cell were found. No significant change in the specific activity of catechol-*O*-methyl transferase was noted throughout the course of the experiment.

The possibility that subculturing procedures affected AChE activity was also investigated. Incubation of stationary phase cells with trypsin resulted in a 30% decrease in cell protein and in AChE activity; thus the specific activity of AChE remained constant. In other experiments not shown here, stationary phase cells were dissociated with trypsin and then plated at the original cell concentration and incubated for 2 days. No change in AChE specific activity was detected throughout the period of incubation. In addition, two cell populations were maintained separately in logarithmic growth by subculturing every other day for 10 days. One population was subcultured by the trypsin procedure, the other with modified D_1. Cells then were incubated for 17 days. Values obtained with cells treated with trypsin did not differ significantly from those obtained with cells passaged with modified D_1 shown in Fig. 1. The results suggests that AChE, but not catechol-*O*-methyl-transferase, responds to a regulatory mechanism that is coupled to the rate of cell division.

Neuroblastoma cells stop dividing in the absence of serum but continue to

synthesize protein.[5] If AChE activity and cell division are inversely related, then removal of serum from the growth medium should result in an increase in AChE activity. The effect of serum concentration on AChE activity and cell growth is shown in Fig. 2. In the presence of serum, cell growth was logarithmic and AChE specific activity remained constant (Fig. 2*A*). In the absence of serum, the cell population quadrupled within 2 days and then remained constant. When cell division was restricted, a marked increase in AChE specific activity was observed (Fig. 2*B*). In this experiment, the cell concentration at the time that AChE specific activity increased was 31,000 cells/cm^2 compared with 200,000 in the experiment illustrated in Fig. 1. Thus regulation of AChE activity is not a function of cell concentration under these conditions.

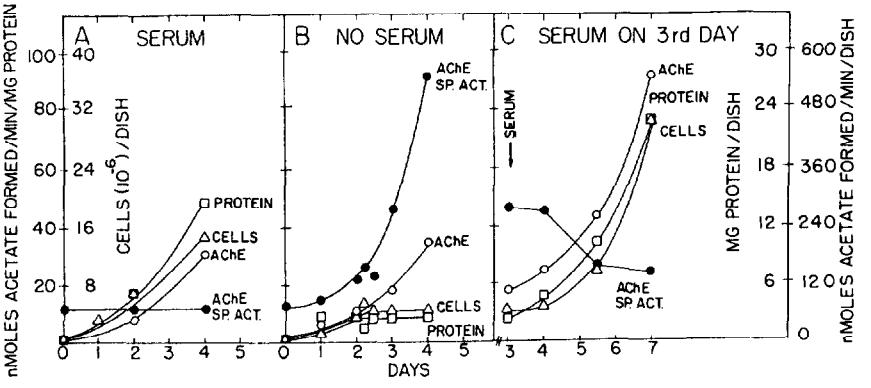

FIG. 2. The rate of cell division was regulated by adjusting the serum concentration. Neuroblastoma cells were incubated for 24 hr prior to zero time in 150-mm dishes containing the medium described, except that horse serum was omitted. At zero time the medium was removed and cells were washed once with growth medium; then fresh medium with or without 10% fetal calf serum was added as specified in *panel A* and *B*, respectively. Some cells were incubated for 3 days without serum, as shown in *panel B;* nondividing cells were then shifted up to the rapidly dividing state by the addition of 10% fetal calf serum (*panel C*). The medium was changed and fresh medium containing 10% fetal calf serum was added at 4 and 5.5 days. Symbols correspond to the following: ●, AChE specific activity; ○, AChE activity/dish; △, cells ($\times 10^{-6}$)/dish; □, mg protein/dish.

Cells were incubated without serum for 3 days (Fig. 2*B*); serum was added on the 3rd day to shift cells from stationary to logarithmic growth without subculturing them (Fig. 2*C*). A decrease in AChE specific activity resulted. The results again show that regulation of AChE activity is coupled to cell division and is not a consequence of subculturing procedures.

Cell division was also restricted by incubating cells with 2 mM thymidine in the presence of serum and growth medium. An increase in AChE specific activity was found (data not shown).

The possibility that the changes in AChE activity were due to activation or inhibition of the enzyme was investigated by mixing homogenates prepared from cells in the logarithmic and stationary phase of growth. Acetylcholinesterase activity was additive; thus no enzyme effector was detected.

The relation between protein synthesis and AChE regulation was studied by determining the effect of cycloheximide upon AChE activity, cell viability, and

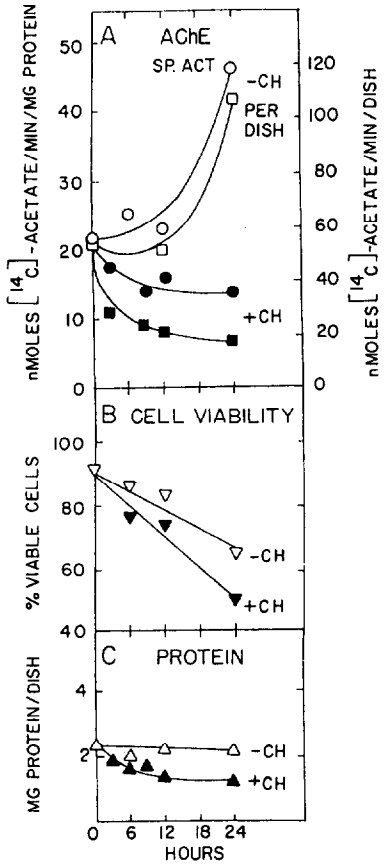

FIG. 3. Neuroblastoma cells were incubated for 2 days without serum, as described in the legend to Fig. 2, to restrict cell division and increase AChE activity. Symbols denote the following: Open symbols, no cycloheximide; closed symbols, 3.5×10^{-5} M cycloheximide added at zero time (48 hr after removal of serum). In *panel A*, AChE specific activity and AChE activity/dish are shown both in the presence and absence of cycloheximide. In *panel B* the percent of cells that were viable and mg of protein/dish are shown, both in the presence and absence of cycloheximide. Each dish contained 4.0×10^6 cells, denoted on the ordinate as 100%. The number of cells/dish did not change significantly during the course of the experiment.

protein (Fig. 3*A*, *B*, and *C* respectively). Cells were incubated without serum for 48 hr before cycloheximide was added. In the absence of cycloheximide, AChE specific activity and units/dish increased 2.5-fold within 24 hr; in the presence of the drug, AChE specific activity and units decreased. The amount of protein and number of viable cells per dish also declined in the presence of cycloheximide. The results suggest that the increase in AChE activity is dependent upon protein synthesis but further data are needed to substantiate this possibility.

Properties of neuroblastoma AChE: A number of enzymes catalyze acetylcholine hydrolysis, including AChE, cholinesterase (EC 3.1.1.8), and acetylesterase (EC 3.1.1.6). The enzymes differ from one another in relative activity towards various substrates and in sensitivity to inhibitors. The effects of selective esterase inhibitors on AChE from stationary phase cells are shown in Fig. 4. Acetylcholine hydrolysis was inhibited 50% in the presence of the following compounds: 1×10^{-8} M diisopropyl fluorophosphate, an inhibitor of AChE, cholinesterase, and carboxylesterase (EC 3.1.1.1), but not arylesterase (EC 3.1.1.2); 6×10^{-8} M 1,5-bis-(4-allyldimethylammoniumphenyl)pentane-1,3-dibromide (BW284C51), a more potent inhibitor of AChE than cholinesterase;[16] 7×10^{-7} M neostigmine sulfate, a more potent inhibitor of AChE or cholinesterase than arylesterase or carboxylesterase; and 4×10^{-4} M tetramonoisopropyl

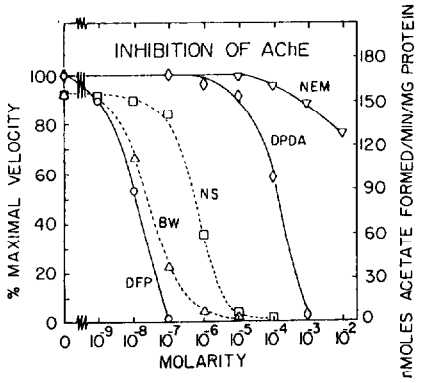

FIG. 4. Effects of esterase inhibitors on AChE from stationary phase neuroblastoma cells. The dotted lines correspond to reactions with a final volume of 0.1 ml, containing the components described and 12.3 μg homogenate protein/reaction, that were incubated at 37°C for 15 min. Abbreviations are as follows: DFP, diisopropylfluorophosphate; BW, 1,5-bis(4-allyldimethylammoniumphenyl)pentane-1,3-dibromide; NS, neostigmine sulfate; DPDA, tetramonoisopropyl pyrophosphortetramide; NEM, *N*-ethylmaleimide.

The solid lines correspond to reactions incubated in two stages. Stage 1 reactions contained the components described in *Materials and Methods* minus [^{14}C]acetylcholine, the esterase inhibitors indicated at 5 times the molarity shown on the abscissa, and 12.3 μg of homogenate protein in a final volume of 20 μl. Stage 1 reactions were incubated for 10 min at 37°C, then placed in an ice bath. Stage 2 reactions were prepared by the addition of 4.13 mM [^{14}C]acetylcholine iodide and other reaction components described so that the final volume of each reaction was 0.1 ml. The final molarity of esterase inhibitors corresponded to that shown on the abscissa. Stage 2 reactions were incubated 15 min at 37°C.

pyrophosphortetramide, a more potent inhibitor of cholinesterase than AChE.[16] *N*-Ethylmaleimide had relatively little effect on the hydrolysis of acetylcholine even at 1×10^{-2} M. In addition, the relative rates of hydrolysis of [^{14}C]-acetylcholine and [^{14}C]butyrylcholine were determined; butyrylcholine hydrolysis was 1% that of acetylcholine. Although definitive characterization awaits the analysis of purified enzyme fractions, the neuroblastoma enzyme appears to be AChE (EC 3.1.1.7).

Discussion. The results show that neuroblastoma acetylcholinesterase activity is regulated and that the regulatory mechanism is inversely related to the rate of cell division. Acetylcholinesterase specific activity increased about 25-fold when the rate of cell division was restricted. Under the same conditions, the specific activity of catechol-*O*-methyl transferase remained relatively constant. Acetylcholinesterase activity in stationary phase neuroblastoma cells was approximately twice that of mammalian brain or erythrocytes, both known to contain relatively high levels of the enzyme.

Studies with neuroblastoma cells revealed that the shift from the dividing to the nondividing state influences the expression of a set of characteristics required for neuron differentiation. Thus far, five properties have been found to increase upon restriction of neuroblastoma cell division: acetylcholinesterase activity, protein per cell, axon-dendrite development, formation of electrically active membranes, and synthesis of acetylcholine receptors.

Neuroblastoma cells are still committed to a program of neuron differentiation even though thousands of cell generations have elapsed since the tumor originated. Commitment to the program may be thought of as the initiating event in the process of differentiation. Once initiated, the progeny apparently do not terminate such a program.

Although the mechanism of initiation is not known, the stability of the phenomenon suggests that it is not fully reversible. It should then be relatively

easy to explore the possibility that normal sympathetic neuroblasts are also irreversibly committed to a pattern of neuronal differentiation.

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Abbreviation: AChE, acetylcholinesterase.

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