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Changes of Ornithine Decarboxylase Activity and Polyamine Content Upon Differentiation of Mouse NB-15 Neuroblastoma Cells

KUANG YU CHEN,* VINCENT PRESEPE, NANCY PARKEN, AND ALICE Y.-C. LIU
Department of Chemistry, Rutgers-The State University of New Jersey, New Brunswick, New Jersey 08903 (K.Y.C., V.P., N.P.), and Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115 (A.Y.-C.L.)

The possible functions of ornithine decarboxylase (ODC) and polyamines in the differentiation of mouse NB-15 neuroblastoma cells were investigated by examining the changes of these parameters in the differentiating and nondifferentiating NB-15 cells over a 5-day culture period. Differentiation of NB-15 cells was induced by the addition of dibutyryl cyclic AMP and 3-isobutyl-1-methylxanthine (IBMX) to the growth medium and was monitored by neurite outgrowth, increases of acetylcholinesterase (AChE), and R_I cAMP-binding protein. Plating of NB-15 cells in fresh serum-containing growth medium was accompanied by rapid growth and a marked increase of ODC activity; this early increase of ODC activity was attenuated, both in duration and in magnitude, in the differentiating cells. The spermidine content of the differentiating neuroblastoma cells was significantly lower than that of the nondifferentiating cells. In the fully differentiated neuroblastoma cells, the ODC activity and spermidine content were lower than that of the undifferentiated cells by approximately 15-fold and five-fold, respectively. Based on these results it is proposed that changes of polyamine metabolism may be of signficance in the differentiation of mouse neuroblastoma cells.

Mouse neuroblastoma cells in tissue culture can be induced to differentiate by the addition of cAMP analogs or agents which increase intracellular cAMP concentration (Prasad and Hsie, 1971; Furmanski et al., 1971). The differentiation can be characterized by the morphological appearance of neurite outgrowth and the biochemical appearance of enzymes involved in neurotransmitter metabolism (for review see Prasad, 1975). Our recent study has also demonstrated that the increase of a 47,000-dalton cAMP-binding protein, R_I, can be used as another biochemical index of differentiation in neuroblastoma cells (Liu et al., 1980). The mechanism by which cAMP triggers differentiation of mouse neuroblastoma cells is not clear. One possible site of cAMP action is the regulation of polyamine metabolism. It has been shown that cAMP analogs and/or cAMP phosphodiesterase inhibitors can enhance ODC (EC 4.1.1.17, L-ornithine carboxy-lyase) activity in animal tissues and cultured cells (Beck et al., 1972; Hogan et al., 1974; Bachrach, 1975; Byus et al., 1976a). In addition, studies of the temporal relationship of ODC activity and cAMP-dependent protein kinase activity have led to the suggestion that the type I cAMP-dependent protein kinase is involved in the induction of ODC (Russell and Stambrook, 1975; Byus et al., 1978; Perchellet and Boutwell, 1980). ODC is the rate-limiting enzyme for the biosynthesis of polyamines (Pegg and Williams-Ashman, 1968). Polyamines are ubiquitous organic cations in living organisms and are implicated in various growth regulatory processes (for recent reviews see Jänne et al., 1977; Russell and Durie, 1978; Canellakis et al., 1979).

Although there are many reports which suggest the causal relationship of elevated ODC activity and polyamine contents to proliferative and neoplastic growth, there have been until recently relatively few studies on the possible involvement of polyamines and ODC in cell differentiation. Stoscheck et al. (1980) have reported a decrease in ODC activity upon fusion of myoblasts to form myotubes. Rath and Reddi (1978) and Takigawa et al. (1981) have demonstrated increases in ODC activity during differentiation of bone marrow cells and rabbit chondrocytes, respectively. A previous study from this laboratory has also demonstrated differences in the mode of regulation of ODC activity in the undifferentiated and differentiated neuroblastoma cells (Chen, 1980). All these studies suggest changes of cellular polyamine contents upon cell differentiation. In the present paper we wish to report our findings on (1) specific changes of basal level of ODC activity and spermidine content during the differentiation of neuroblastoma cells, and (2) the temporal relationship of these changes and the expression of differentiated phenotypes of neuroblastoma cells. The significance of the cAMP-induced changes in ODC activity and polyamine contents in cell differentiation will be discussed.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle medium, fetal calf serum, and dithiothreitol were obtained from Gibco, Grand Island, NY. Three-isobutyl-1-methylxanthine (IBMX), a cAMP-phosphodiesterase inhibitor, was from Aldrich, Milwaukee, WI. Dibutyryl cyclic AMP (Bt_2cAMP), putrescine, spermidine,

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*To whom reprint requests/correspondence should be addressed.

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spermine, L-ornithine, 5-dimethyl amino-1-naphthalene sulfonyl chloride (dansylchloride), and phenylmethylsulfonyl fluoride were purchased from Sigma Chemical Co., St. Louis, MO. L-[1-^{14}C]ornithine monohydrochloride (59 mCi/mmole) and 1-[^{14}C]acetylcholine (20 mCi/mmole) were from Amersham, Arlington, IL, 8-N_3-[^{32}P]cAMP (50 ~ 80 Ci/mmole) was from ICN, Irvine, CA. HPLC grade solvents were from Baker Chemical Co. (Phillipsburg, NJ.) Other chemicals were of standard reagent grade.

Cell culture and differentiation

Mouse NB-15 neuroblastoma cells (a gift of Dr. W. Gibson, Rutgers University) were grown as monolayer culture in Dulbecco's modified Eagle medium (with 4,500 mg glucose per liter, without sodium pyruvate) supplemented with 10% fetal calf serum. Cells were maintained at 37°C in a water-jacketed CO_2 incubator (95% air, 5% CO_2). In view of the consideration that the action of Bt_2cAMP and IBMX in inducing differentiation of the mouse neuroblastoma cells was dependent on the density of the cell population used, such that Bt_2cAMP and IBMX were without effect in promoting the expression of differentiated phenotypes when added to confluent cultures of neuroblastoma cells (data not shown), Bt_2cAMP (1 mM) and IBMX (0.5 mM) were added to sparse cultures of NB-15 neuroblastoma cells at 15 hours after subculture (seeding density 2 $\times$ 10^4 cells/cm^2).

For the time-course experiments, the time zero samples were collected immediately after the addition of Bt_2cAMP and IBMX. Cells grown in the normal growth medium (Dulbecco's medium plus 10% fetal calf serum) will be designated as NB cells, denoting nondifferentiating neuroblastoma cells, whereas cells grown in the "differentiation" medium (i.e. normal growth medium plus 1 mM Bt_2cAMP and 0.5 mM IBMX) will be designated as ND cells denoting differentiating neuroblastoma cells. As illustrated in Figure 1, the doubling time of NB and ND cells was 24 and 36 hours, respectively. When seeded at 2 $\times$ 10^4 cells/cm^2, NB cells reached stationary phase of growth at approximately t = 80 hours with a saturation density of about 3 ~ 4 $\times$ 10^5 cells/cm^2; ND cells reached stationary phase of growth at approximately t = 70 hours with a saturation density of about 0.8 ~ 1 $\times$ 10^5 cells/cm^2. It should be noted that while the growth rate at the logarithmic phase and the saturation density at the stationary phase of growth of the ND cells were lower than that of the NB cells, the mere inhibition of cell growth was not sufficient to induce the differentiation of neuroblastoma cells (Schubert et al., 1971; Liu et al., 1981). The term differentiated neuroblastoma cells will be used to refer to the ND cells that have entered the stationary phase of growth (t = 70 ~ 105 hours).

Cell harvesting

At various time intervals, cells grown in 60-mm culture dishes were quickly washed with phosphate-buffered saline. Cells were then scraped off the substratum with a rubber policeman and processed according to the following procedures. For determination of ODC activity and polyamine contents, cells were harvested in a buffer containing 0.1 mM EDTA, 50 μM pyridoxal phosphate, and 5 mM dithiothreitol in 50 mM Tris-HCl (pH 7.4 at 22°C). For determination of AChE and R_1 cAMP-binding activities, cells were first harvested in phosphate-buffered saline,

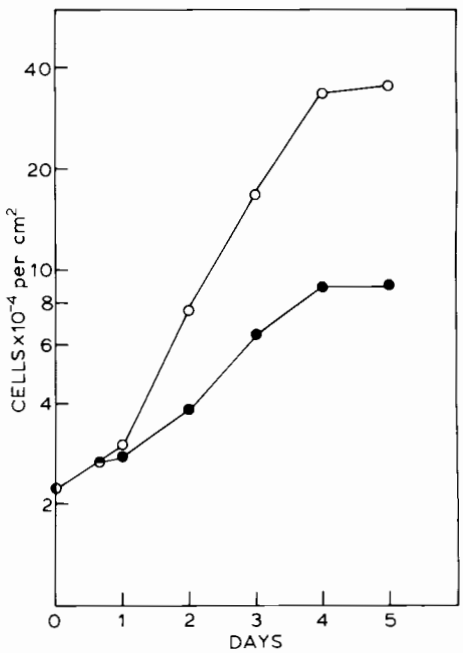

Fig. 1. Growth rates of control (NB) (—○—) and differentiating (ND) (—●—) NB-15 mouse neuroblastoma cells. NB-15 neuroblastoma cells were seeded at a density of 2 $\times$ 10^4 cells/cm^2 in 60-mm tissue culture dishes. To induce differentiation of the NB-15 cells, Bt_2cAMP (1 mM) and IBMX (0.5 mM) were added at 15 hours after subculture. Day 0 denotes the time of subculture.

sedimented by centrifugation, resuspended, and sonicated in a buffer of 10 mM Tris-HCl (pH 7.4 at 22°C) containing 0.1 mM EDTA and 50 μg/ml phenylmethylsulfonyl fluoride. The cell homogenate or a 100,000 $\times$ g supernatant fraction of the homogenate (cytosol) was used for determination of enzyme activities.

Assay of ODC activity

ODC activity was determined using a 12,000 $\times$ g supernatant obtained from cell homogenate according to procedures described previously (Chen et al., 1976) One unit of ODC activity is defined as 1 nmole CO_2 evolved per hour. Protein concentration was determined by a modified Lowry's method with bovine serum albumin as the standard (Ross and Shatz, 1973).

Quantitation of individual polyamine content

The methods of Seiler and Wiechman (1970) of preparing dansyl derivatives of polyamines and of the extraction and analysis of these dansylated polyamines by high-pressure liquid chromatography (HPLC) were used. Briefly, cell homogenates were deproteinized by the addition of perchloric acid (final concentration 0.2 M), incubated at 4°C for 2 hours, and precipitates removed by centrifugation. To a 400-μl aliquot of the perchloric acid extract, 25 μl of saturated Na_2CO_3 solution and 300 μl of saturated $NaHCO_3$ solution were added to bring the pH to 9.5. Dansylation was initiated by adding 500 μl of dansyl chloride (10 mM dissolved in acetone) to this solution and was carried out at room temperature in the dark for 15 hours. Excess dansyl

chloride was then removed by adding 10 μl of a proline solution (1.5 M) and incubated for 20 minutes. Acetone present in the samples was evaporated under nitrogen stream. The dansylated polyamines were then extracted from the aqueous phase with 1 ml of toluene. The mixture was centrifuged at 500 × g for 10 minutes, 0.8 ml of the toluene layer was transferred to a 12 × 75-mm culture tube and dried at 45°C in a vacuum oven and reconstituted with an appropriate volume of methanol (HPLC grade). The dansylated polyamines were then separated on a reverse-phase column (RP-18, 7 μm ODS column, Unimetrics Corp.) connected to a Beckman model 110A pump. A Schoeffel spectrofluoromonitor (Model FS 970) was used to detect the dansylated polyamines and an Integrating Chart Recorder (Linear Instruments Corp.) was used to record the chromatogram. The solvent system used was acetonitrile-H_2O (85:15, v:v). The amounts of cellular polyamines were calculated by comparing their peak areas with standard samples which were dansylated and extracted under identical experimental conditions. The retention times of putrescine, spermidine,and spermine were 2.1, 3.4, and 6.7 minutes respectively at a flow rate of 2.3 ml/minute.

Quantitation of R_I cAMP-binding protein

Photoactivated incorporation of 8-N_3-[^{32}P]cAMP was used to quantitate R_I cAMP-binding protein and was carried out according to methods previously described (Liu et al., 1980). The standard reaction mixture (final volume 0.1 ml) contained 50 mM 2-(N-morpholino)ethanesulfonate (MES) (pH 6.2), 10 mM $MgCl_2$, 50 μM ATP, 1 μM 8-N_3-[^{32}P]cAMP (specific activity 4 ~ 10 Ci/mmole) and approximately 50–200 μg of cytosol protein. Samples were incubated for 60 minutes at 4°C in the dark and were then photolyzed for 10 minutes with a mineralite UVS-11 hand lamp at a distance of 8 cm. The amount of 8-N_3-[^{32}P]cAMP incorporated was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography.

Assay of acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was determined by the radiometric method described by Ehrenpreis et al. (1970); results are expressed in nmoles of 1-[^{14}C]acetate generated per minute per mg protein for a substrate concentration of 10^{-4} M.

RESULTS

Ornithine decarboxylase activity of the NB and ND cells was studied as a function of time in tissue culture over a 5-day period. Results are shown in Figure 2. In the NB cells, there was a remarkable early increase in ODC activity. This early increase in ODC activity was temporally correlated with the resumption of logarithmic growth after plating; the result is in agreement with the notion of a close association of intracellular ODC activity and rapid cell growth. The increase in ODC activity in NB cells was sustained for the time period of 27 to 77 hours in tissue culture and may consist of two partially resolved peaks (2.8 units/mg protein and 3.2 units/mg protein at t = 35 and 70 hours, respectively). Upon entering the stationary phase of cell growth, the ODC activity of NB cells decreased precipitously, reaching a minimal activity of 0.2 unit/mg protein at the end of the 5-day culture period. The time course of change in ODC activity of the ND cells differed from

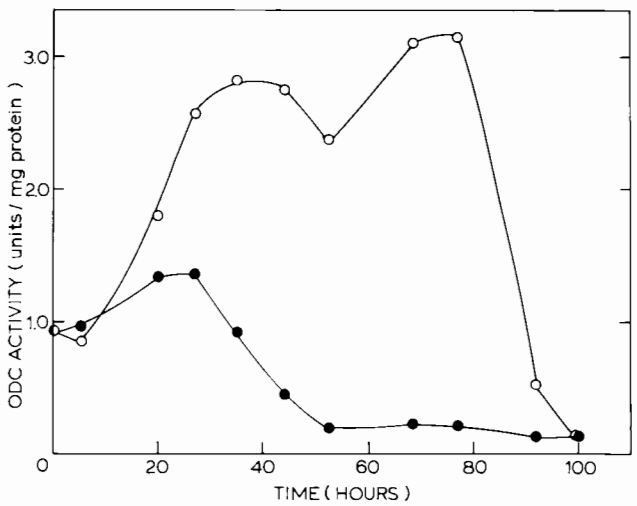

Fig. 2. Changes of ornithine decarboxylase (ODC) activity in NB (–○–) and ND (–●–) cells as a function of time in tissue culture. Time zero refers to the time when Bt_2cAMP (1 mM) and IBMX (0,5 mM) were added to the ND culture (15 hours after plating). ODC activity was determined as described under Materials and Methods. Results shown are representative of three separate experiments.

that of the NB cells in two respects. First, the early increase in ODC activity of the ND cells was attenuated in both magnitude and duration when compared to that of the NB cells. Maximal ODC activity of the ND cells, being 1.4 units/mg protein, was obtained at t = 24 hours. Second, the increase in ODC activity of the ND cells was transient; a steady decline in ODC activity was observed thereafter, reaching a minimal value of 0.2 units/mg protein at t = 50 hours, and remained at that level for the remaining culture period.

ODC is the rate-controlling enzyme for the biosynthesis of polyamines. In view of this consideration and to gain a better understanding of the possible role of polyamines in neuroblastoma cell differentiation, the cellular contents of putrescine, spermidine, and spermine of NB and ND cells, at various times during the 5-day culture period, were measured by the HPLC method as described. Results of such an experiment are shown in Figure 3.

Putrescine appeared to be present in lower concentration than either spermine or spermidine (Fig. 3A). In addition, there was no significant difference in the putrescine content of the NB and ND cells; in both cases, the level of putrescine decreased slightly as a function of time in tissue culture. The results obtained on putrescine levels are in agreement with studies previously reported (Kremzner et al., 1975; Rupniak and Paul, 1978).

Measurement of cellular spermidine content revealed significant differences between the NB and the ND cells (Fig. 3B). The spermidine content of the NB cells followed a steady increase from the basal level of 1.5 nmoles/mg protein (at t = 27 hours) to a maximal value of 5.05 nmoles/mg protein (at t = 70 hours). The elevated level of spermidine of NB cells was sustained for the remainder of the 5-day culture period. The pattern of change in spermidine content of the ND cells was distinct from that of the NB cells.

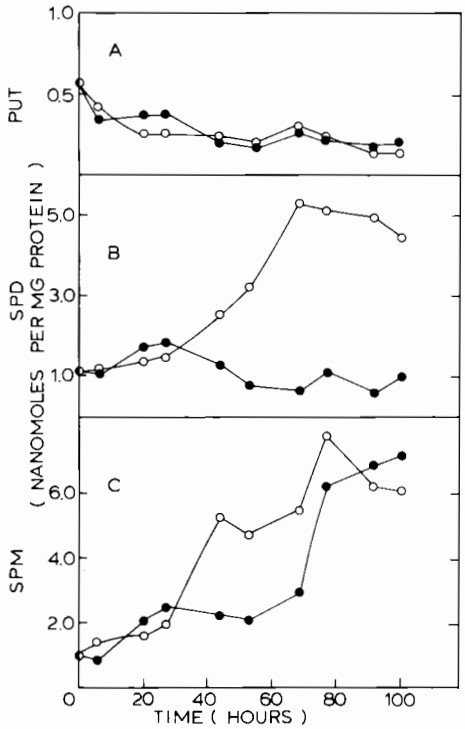

Fig. 3. Changes of polyamine contents in NB (−○−) and ND (−●−) cells as a function of time in tissue culture. Time zero refers to the time when Bt_2cAMP and IBMX were added to ND culture (15 hours after plating). A. Comparison of putrescine content between NB and ND cells. B. Comparison of spermidine content between NB and ND cells. C. Comparison of spermine content between NB and ND cells. Results shown are representative of three separate experiments.

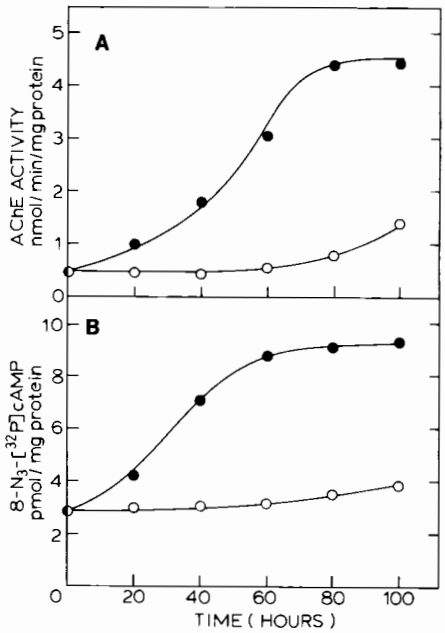

Fig. 4. Cytosolic acetylcholinesterase activity (A) and incorporation of 8-N_3-[^{32}P]cAMP into the 47,000-d R_IcAMP-binding protein (B) of NB (−○−) and ND (●) cells. Time zero refers to 15 hours after plating when Bt_2cAMP and IBMX were added to the ND culture to initiate differentiation. All assays were performed according to methods described. Results shown are representative of two separate experiments.

A small peak of spermidine (1.8 nmoles/mg protein) was observed in the ND cells during the early log phase of cell growth; it may be noteworthy that the time course of increase in spermidine content of the ND cells correlates well with the time course of increase of ODC activity in these cells.

In both the NB and ND cells, there appeared to be a progressive increase in the spermine content as a function of growth in tissue culture (Fig. 3C), although the increase in spermine of the NB cells appeared to precede that of the ND cells. The highest concentration of spermine achieved in the NB cells was comparable to that of the ND cells.

To try to relate the changes of ODC activity and polyamine contents to the expression of other differentiated phenotypes of neuroblastoma cells, we also measured the time course of changes of AChE and R_I cAMP-binding activities in both NB and ND cells over the 5-day culture period (Fig. 4).

In agreement with our previous findings of the N-18 mouse neuroblastoma cells (Liu et al., 1980), both the AChE and R_I cAMP-binding activity increased gradually in the differentiating cells treated with Bt_2cAMP + IBMX, reaching maximal values when cells entered the stationary phase of growth. By comparing the results illustrated in Figures 2, 3, and 4, it can be noted that when the two differentiated phenotypes, AChE and R_I cAMP-binding protein, were maximally expressed (t = 70–105 hours), the differences in ODC activity and spermidine content between NB and ND cells were also the greatest.

DISCUSSION

The ability of cAMP or agents which increase intracellular cAMP concentration in inducing the differentiation of mouse neuroblastoma cells has been well documented (Prasad, 1975). Thus, addition of Bt_2cAMP and IBMX to mouse neuroblastoma cells triggers the formation of neurites and the synthesis of neurospecific enzymes. The molecular mechanism of cAMP-induced cell differentiation is of general interest because cAMP is able to induce differentiation of many other cell types such as melanoma cells (Johnson and Pastan, 1972), muscle cells (Zalin and Montague, 1974), and chondrocytes (Takigawa et al., 1981), in addition to that of neuroblastoma cells. Although the precise mechanisms of action of cAMP in cellular differentiation are not well understood, the involvement of cellular ODC activity and polyamines seems likely. Previous studies have demonstrated the ability of cAMP in inducing ODC activity (Beck et al., 1972; Hogan et al., 1974; Bachrach, 1975; Byus et al., 1976b). Furthermore, the roles of ODC and polyamines in cell growth regulation are widely accepted (Cohen, 1971; Bachrach, 1973; Russell and Durie, 1978). In this report we studied the changes of ODC and polyamines in NB and ND cells. Our results indicated that differentiation of mouse neuroblastoma cells was accompanied by (1) an attenuation of the early peak of ODC activity, and (2) a lowered spermidine content when compared to that of the undifferentiated cells. Maximal differences of both ODC activity and spermidine content

in the NB and ND cells occurred at t = 70–80 hours and are temporally correlated with the maximal expression of AChE and R_I cAMP-binding protein in the ND cells.

The findings of an early attenuation of ODC activity after the addition of Bt_2cAMP and IBMX to sparse cultures of neuroblastoma cells may seem to contradict the ability of cAMP analogs and/or cAMP phosphodiesterase inhibitor to induce ODC activity in confluent mouse neuroblastoma cells (Bachrach, 1975; Chen and Canellakis, 1977). This dichotomous effect of cAMP in neuroblastoma cells appeared to be related to cell growth/cell density. Thus, in rapidly dividing sparse cultures of neuroblastoma cells (cell density 2×10^4 cells/cm^2 or less) the addition of cAMP analogs and/or cAMP phosphodiesterase inhibitors results in, in chronological order, neurite outgrowth within 4 hours, attenuation of ODC activity, slowing of cell growth, and maximal expression of differentiated phenotypes. On the other hand, the addition of cAMP to confluent cultures of neuroblastoma cells (cell density = $4 \sim 6 \times 10^5$ cells/cm^2), while eliciting a greater then 100-fold increase in ODC acitivity, was ineffective in inducing the expression of differentiated phenotypes in these cells (data not shown).

The ability of cAMP to induce ODC activity of hepatoma (Byus et al., 1976b), glioma (Bachrach, 1975), and BHK cells (Hogan et al., 1974) has previously been reported. By contrast, cAMP decreases ODC activity and causes growth arrest of the S-49 mouse lymphoma cells (Insel and Fenno, 1978; Kaiser et al., 1979). In the case of Y1 adrenocortical tumor cells, the addition of cAMP results in an increase in ODC activity while inhibiting the growth of these cells (Kudlow et al., 1980). These observations and the results presented in this study suggest a potentially complex relationship of cAMP and ODC activity, one which may be dependent on or modulated by the status of growth/differentiation of the cells and perhaps also the cell type used.

Although polyamines have been implicated in many growth regulatory processes, the precise function of the individual polyamines remains to be elucidated. Of the three polyamines (putrescine, spermidine, and spermine), the marked increase of spermidine content in the NB cells upon reaching a stationary phase of cell growth and the lack of such increase in the ND cells appears to be the most notable difference in polyamine metabolism between these two cell populations. The content of spermine increased as a function of time in both the NB and ND cells; furthermore, the large increase of spermine in ND cells at t = 70 hours was temporally related to the maximal expression of AChE and R_I cAMP-binding activities.

In studying the role of polyamines in cellular processes, it appears likely that the ratio of relative concentrations of polyamines may be of significance. For example, it has been observed that a high spermidine/spermine ratio is typical for a tissue undergoing rapid growth or hypertrophy whereas low spermidine/spermine ratio is generally typical of a tissue with low biosynthetic activity or of differentiated tissues with constant rate of RNA and protein synthesis (Jänne et al., 1964; Russell and Durie, 1978). In this regard, the calculated molar ratio of spermidine/spermine of the NB and ND cells at the stationary phase of growth (t = 70–80 hours) were 0.96 and 0.2, respectively.

In attempting to delineate the functions of polyamines in the differentiation of neuroblastoma cells, one possible approach is to either inhibit or augment the cellular contents of polyamines. To this end, we have obtained preliminary evidence that the inhibition of spermidine accumulation may lead to differentiation of neuroblastoma cells[1].

While the data presented in this report may not estabish a definitive cause-effect relationship between the alterations of ODC activity/polyamine contents and differentiation of neuroblastoma cells, they do suggest that changes of polyamine metabolism, such as the early attenuation of ODC activity, the decrease of spermidine level, and the late increase of spermine, may be involved in the biochemical pathways which lead to the full expressions of differentiated phenotypes of mouse neuroblastoma cells.

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