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Differentiation of a mouse neuroblastoma variant cell line whose ornithine decarboxylase gene has been amplified

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Differentiation of mouse neuroblastoma cells has been shown to be accompanied by changes in polyamine metabolism and a decrease in polyamine content. We have previously shown that α-difluoromethyl ornithine, a suicide inhibitor of ornithine decarboxylase (ODC, EC 4.1.1.17) and suboptimal concentrations of dibutyryl cAMP (0.1 to 0.2 mM) are effective in inducing the differentiation of mouse Neuro-2a (N2a) neuroblastoma cells. Exogenously added putrescine or spermidine can block the action of DFMO and dibutyryl cAMP, suggesting that polyamines may play a regulatory role in neuroblastoma differentiation. We have now isolated from N2a cells a clonal variant line, DF-40, whose ODC gene has been amplified by 40-fold. The DF-40 cells overproduced the ODC enzyme and contained very high levels of putrescine, spermidine and spermine. Treatment of DF-40 cells with dibutyryl cAMP or DFMO/dibutyryl cAMP led to a more than 80% reduction in polyamine content. Such a decrease did not cause the DF-40 cells to differentiate. Polyamine content in the treated DF-40 cells was still comparable or higher than that in the undifferentiated N2a cells. In contrast, serum-deprivation induced full differentiation of DF-40 cells. Levels of polyamine in the differentiated DF-40 cells, however, were also found to be comparable to that in the undifferentiated N2a cells. Exogenously added polyamines could not block the differentiation of DF-40 cells induced by serum-deprivation, suggesting that the action of polyamines in regulating neuroblastoma differentiation may depend on the presence of serum factors.

Introduction

Ornithine decarboxylase (ODC, EC 4.1.1.17) is the key enzyme in the biosynthesis of polyamines [1]. The modulation of ODC activity and polyamine content by inhibitors for polyamine biosynthesis has been shown to affect the growth and differentiation of many cultured cell lines [2–4]. We have previously shown that α-fluoromethyl ornithine (FMO) and α-difluoromethyl ornithine (DFMO), both catalytic irreversible inhibitors of ornithine decarboxylase, promote the differentiation of mouse neuroblastoma cells [5]. Others have reported that DFMO can induce the differentiation of mouse melanoma cells [6,7], mouse teratocarcinoma cells [8] and embryonic carcinoma cells [9]. The differentiation of these tumor cells in vitro has been shown to be accompanied by a reduction in polyamine content. The inhibitor DFMO can also be used to generate ODC overproducers with the ODC gene amplified by selecting cells for resistance to high concentrations of DFMO [10–13]. These ODC overproducers have been used extensively to study the molecular biology and regulation of the ODC gene. We reasoned that if the manipulation of the polyamine content in mouse neuroblastoma cells can lead to their differentiation [5,14], it may be difficult or impossible to induce the differentiation of the mouse neuroblastoma ODC overproducers due to high intracellular polyamine content. To test this hypothesis, we have isolated and characterized a variant clonal cell line, DF-40, derived from the parental N2a mouse neuroblastoma cell line. This DF-40 cell line was resistant to DFMO upto 40 mM and exhibited a doubling time comparable to that of N2a cells even when grown in the presence or absence of 40 mM DFMO. We examined the ability of the DF-40 cells to differentiate under three different conditions which are known to induce the full differen-

Abbreviations: ODC, ornithine decarboxylase; DFMO, α-difluoromethyl ornithine; FMO, α-monofluoromethyl ornithine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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tiation of the parental N2a mouse neuroblastoma cells. We found that although dibutyryl cAMP and DFMO/dibutyryl cAMP caused, respectively, 95% and 80% suppression of the polyamine content in DF-40 cells, both treatments were ineffective in inducing the differentiation of DF-40 cells. In contrast, serum-deprivation was found to be effective in eliciting differentiation of DF-40 cells. The polyamine level in the serum-deprived DF-40 cells was reduced by 95%, comparable to that in the DF-40 cells treated with dibutyryl cAMP. Exogenously added putrescine, spermidine or spermine could not block the differentiation of DF-40 cells induced by serum-deprivation. Taken together, our data suggest that the correlation between polyamine contents and the differentiated phenotypes of mouse neuroblastoma cells may depend on the presence of serum. It is possible that the cellular polyamines may be sequestered when cells are maintained in the absence of serum and thus not available for regulating growth and differentiation. It is also possible that certain serum factors may be needed in order for the cellular polyamines to counteract the effects of differentiation-inducing agents such as cAMP.

Materials and Methods

Materials. All tissue culture supplies were obtained from Gibco, Grand Island, NY. Biochemicals were purchased from Sigma, St. Louis, MO. Phenol was from Research Orangic Inc., Cleveland, OH; chloroform, 2-propanol and formaldehyde were from Fisher, Springfield, NJ. [1-^{14}C]Acetylcholine chloride (56 mCi/mmol) was from Amersham, Arlington, IL, EN^{3}HANCER, L-[1-^{14}C]ornithine (54.3 mCi/mmol), [5-^{3}H]DFMO (39.2 Ci/mmol) and GeneScreen Plus membrane were purchased from New England Nuclear, Boston, MA. Sodium dodecyl sulfate (SDS), agarose and [α-^{32}P]dCTP (3000 Ci/mmol) were from ICN Chemical Radioisotope Division, Irvine, CA. *Eco*RI, *Hin*dIII, *Bam*HI, RNase, proteinase K and the nick translational kit were from Bethesda Research Laboratories, Bethesda, MD. DFMO was a generous gift from Dr. P.P. McCann, Merrell Dow Research Institute, Cincinnati, OH. High-pressure liquid chromatography grade solvents were purchased from Baker, Phillipsburg, NJ. The plasmid pODC54 containing ODC cDNA was kindly given to us by Dr. O. Janne [15], Rockefeller University, NY.

Cell culture. Mouse Neuro-2a (N2a) neuroblastoma cells were maintained and grown in Dulbecco's modified Eagle's medium (with 4500 mg glucose per l) supplemented with 10% fetal bovine serum as previously described [5]. The DFMO-resistant cells were obtained by growing N2a cells in increasing concentrations of DFMO starting with 0.05 mM and proceeding through to 0.1, 0.5, 3, 5, 9, 12, 20, 25, 30 and 40 mM. When DFMO concentrations in the culture exceeded 20 mM, the amount of NaCl in the medium was reduced correspondingly in order to maintain the isotonicity of the growth medium. The cells were maintained at each concentration of DFMO until their growth rate became the same as control N2a cells in the normal growth medium. Cells resistant to 9 mM, 20 mM and 40 mM DFMO were cloned by limiting dilution as described in Results. The clonal cell line resistant to 40 mM was named as DF-40 mouse neuroblastoma cells and used for characterization and subsequent physiological studies.

Characterization of DF-40 cells mouse neuroblastoma cells. High molecular weight cellular DNA was isolated by the procedure described by Arrand [16]. The DNA was digested with three different restriction endonucleases as indicated in the figure legends. Southern blot analysis was performed on a 0.8% agarose gel and the nick-translated radioactive pODC54 was used for hybridization. Total cellular RNA was isolated by the method of Chomczynski and Sacchi [17]. Poly(A)$^+$ RNA was prepared from the total RNA by oligo(dT) cellulose affinity chromatography. RNA blot analysis was carried out on a 1% agarose gels containing formaldehyde, transferred onto a GeneScreen Plus membrane and hybridized to a nick-translated pODC54. The amount of active ODC molecules in the N2a and DF-40 cells were compared by the radioactive affinity labeling technique developed by Seely and Pegg [18]. Briefly, confluent mouse neuroblastoma cells were washed three times with phosphate-buffered saline, then harvested in a buffer containing 0.05 M Tris-HCl (pH 7.3), 0.05 mM pyridoxal phosphate, 0.1 mM EDTA and 5 mM dithiothreitol. Samples were homogenized by sonication and centrifuged at $12000 \times g$, 4°C for 6 min. The supernatants (200 μg) were incubated with 6 μCi [5-^{3}H]DFMO (39.2 Ci/mmol) at 37°C for 5 h and dialyzed overnight in a buffer containing 50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 1 mM dithiothreitol and 0.02% Brij 35. The labeled samples were analyzed by SDS-PAGE and fluorography.

Differentiation of the N2a and the mutant DF-40 mouse neuroblastoma cells. Three conditions, namely, serum-deprivation, the addition of dibutyryl cAMP and the addition of DFMO plus dibutyryl cAMP were tested for their ability to induce the differentiation of DF-40 mouse neuroblastoma cells. All three conditions have been shown to be effective in causing both the morphological and biochemical differentiation in N2a and other mouse neuroblastoma cell lines [5,14]. Serum-deprivation was initiated 5 to 10 h after subculturing cells at a seeding density of about $2 \cdot 10^4$ cells/cm^2 by replenishing the complete serum-containing growth medium with serum-free Dulbecco's medium. DFMO (200 mM) and dibutyryl cAMP (100 mM) stock solutions were prepared fresh in sterile

water before use and were added to the cell culture dishes immediately after subculturing in Dulbecco's medium plus 10% fetal bovine serum at the concentrations indicated in the figure legends. The seeding density was approx. $1 \cdot 10^4$ cells/cm^2.

Biochemical assays. ODC activity in the cell lysates was determined according to the procedures described previously [14]. One unit of ODC activity is defined as the release of 1 nmol of CO_2 from ornithine per h at 37°C. Cellular polyamine contents were quantitated by a pre-column derivatization with dansyl chloride and HPLC separation using a 5 mm octadecylsilane reverse-phase column as was previously described [5,14]. Acetylcholinesterase activity present in the neuroblastoma cell extracts was determined by a radiometric assay method according to the procedure described previously [5,14]. The results are expressed in nmol of acetylcholine hydrolyzed per min per mg protein at 37°C with a substrate concentration of $2.5 \cdot 10^{-4}$ M. Protein concentration was determined by a modified Lowry's method using bovine serum albumin as the standard [19].

Results

Selection of DFMO-resistant mouse neuroblastoma cells

The stepwise method used for producing a dihydrofolate reductase overproducer [20] was adopted to generate mouse neuroblastoma variants which may overproduce ODC. A similar approach has been used to generate ODC overproducers from other tumor cell lines [10–12]. Since DFMO inhibits the growth of N2a mouse neuroblastoma cells at concentrations between 20 μM and 50 μM [5], the selection of DFMO-resistant mutant cells was initiated at 0.05 mM of DFMO and the concentrations of DFMO were increased through 0.1, 0.5, 3, 5, 9, 12, 20, 25, 30, until 40 mM. Cells were maintained at each of the above concentrations for days or weeks until their growth rate became similar to that of the N2a parental cells. Clonal cell lines were isolated from resistant cells grown at 9, 12, 20, 30 and 40 mM DFMO by seeding approx. 1000 cells in a 100 mm culture dish containing semisolid agar (0.5%) medium made of normal growth medium and the appropriate concentration of DFMO. The clones were then expanded sequentially in 96, 24 and 6-multiwell plates. The DF-40 clonal line has been maintained in culture in the absence of DFMO for more than 2 years without losing its resistance to DFMO. The DF-40 cells grown in the absence or presence of DFMO exhibited a comparable growth rate and morphology as the parental N2a cells (data not shown).

Induction of ODC activities by serum in DFMO-resistant cells

The activity of ODC in mouse neuroblastoma cells can be induced by replenishing the medium of confluent cultures with fresh serum-containing growth medium [21]. Fig. 1 shows that the serum-induced ODC activity in DFMO-resistant cells correlated well with their resistance to DFMO up to 30 mM. The maximal ODC activity induced by serum in N2a, DF-9, DF-12 and DF-30 cells was, respectively, 4, 12, 25 and 60 units/mg protein. A dramatic increase in both the basal and induced levels of ODC activity occurred in DFMO-resistant cells only when the concentration of DFMO in the selection medium reached 40 mM. Thus, the maximal ODC activity in DF-40 cells was found to be 360 units/mg protein, 90-fold greater than that in N2a cells.

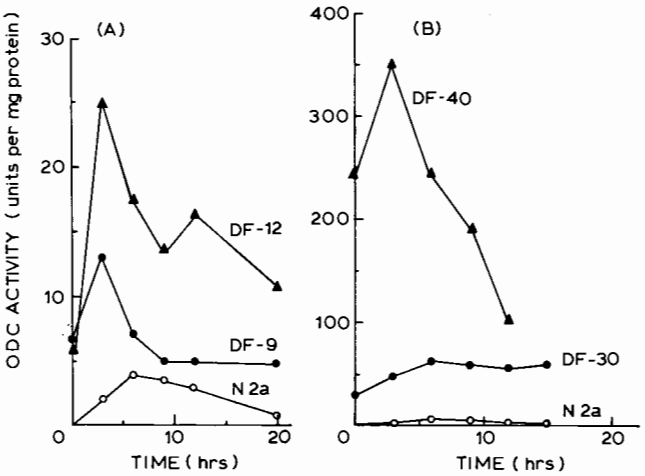

Fig. 1. The induction of ornithine decarboxylase (ODC) activity induced by serum in N2a and various DFMO-resistant mouse neuroblastoma cell lines. N2a and all DFMO-resistant cells in cultures at a 90% confluence of growth were serum-deprived for 22 h and then subject to serum stimulation by replenishing the media with fresh Dulbecco's medium and 10% fetal bovine serum. Cells were harvested at various time points thereafter for ODC assay. All DFMO-resistant cells were maintained in cultures in the absence of the drug for 2 weeks prior to the experiment. The time-course of ODC induction in N2a cells was compared to that in DF-9 and DF-12 cells in A and to that in DF-30 and DF-40 cells in B. Notice the difference in the scale for ODC activity.

Characterization of the DF-40 clonal cell line

DFMO-resistance could be due to any one of or several different mechanisms including alteration of the transport system for the drug uptake, alteration of the target enzyme or gene amplification. The total uptake of DFMO in the confluent culture of DF-40 cells was about 20% less than that in N2a cells (data not shown). Such a small decrease in uptake could not account for the high resistance of DF-40 cells to DFMO. Southern blot analysis, shown in Fig. 2A, provided evidence that amplification of the ODC gene did occur in DF-40 cells, similar to that reported for other tumor cell lines [10–12]. The size of the *Eco*RI, *Bam*HI and *Hin*dIII restriction fragments shown in Fig. 2A for mouse neuroblastoma cells were similar to

that reported for mouse myeloma cells [22] and S49 mouse lymphoma cells [23]. Based on the hybridization intensity of the restriction fragments shown in Fig. 2A, we estimated that the ODC gene in DF-40 cells has been amplified by about 40-fold. Among the three *Hin*dIII fragments shown in the autoradiogram, only the 2.0 kb one was clearly amplified, suggesting that ODC may belong to a multigene family as previously reported for the mouse S49 overproducer [23]. Northern blot analysis shown in Fig. 2B indicated that the sizes of ODC mRNA in both N2a and DF-40 cells were about 2.2 kb, similar to that reported for other rodent cells [15,22]. The basal level of ODC mRNA in DF-40 cells was more than 80-fold higher than that in N2a cells induced by serum (Fig. 2B). The difference in the ODC activity between N2a and DF-40 cells can be accounted for by the difference in the amount of active ODC molecules as shown in Fig. 2C. The overproduction of ODC mRNA and protein was also reflected in the large increase in polyamine content in DF-40 cells as shown in Table I. When both N2a and DF-40 cell cultures at 70% confluency were compared, the putrescine, spermidine and spermine contents in DF-40 cells were found to be, respectively, 215-fold, 30-fold and 14-fold higher than those in N2a cells. We also included DF-12 cells in this study for comparative purposes. It is interesting to note that the polyamine content in DF-12 cells did not differ significantly from that in N2a cells.

TABLE I

Polyamine content in N2a and DFMO-resistant mouse neuroblastoma cells

Contents (nmol/mg protein) [a]	N2a	DF-12	DF-40
Putrescine	0.90±0.02	6.2±0.1	194±10
Spermidine	10.7 ±0.2	9.5±1.2	323± 9
Spermine	9.8 ±1.4	12.1±0.3	133± 7
Total polyamines	21.4	27.8	650

[a] N2a, DF-12 and DF-40 mouse neuroblastoma cells were grown to 75% confluency in culture dishes and harvested for polyamine quantitation and protein determination.

Differentiation of N2a and DF-40 mouse neuroblastoma cells

Three different conditions, namely, dibutyryl cAMP treatment, DFMO/dibutyryl cAMP treatment and

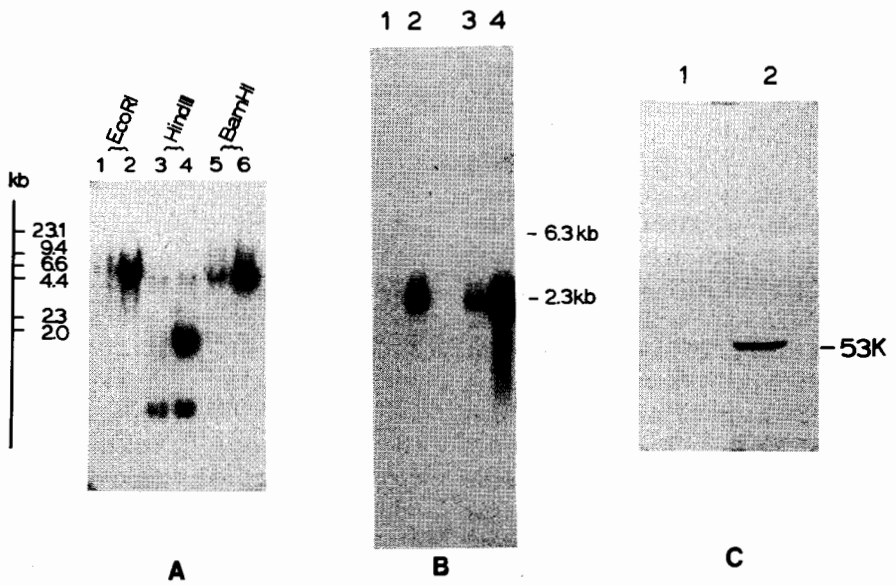

Fig. 2. Characterization of DF-40 mouse neuroblastoma ODC overproducer cell line. (A) Southern blot analysis of DNA from N2a and DF-40 cells. Genomic DNA from N2a (lanes 1, 3 and 5) and DF-40 cells (lanes 2, 4 and 6) were isolated, digested with endonucleases as indicated, fractionated and then transferred onto a GeneScreen plus membrane. DNA fragments homologus to ODC gene sequence were detected by hybridization of the membrane to the ^{32}P-labeled pODC54 probe and further autoradiography. *Hin*dIII digest of the lambda DNA was used as DNA size standards. (B) Northern blot analysis of ODC gene expression in N2a and DF-40 cells. Total RNA and poly(A)$^+$ RNA were isolated, fractionated by formaldehyde/agarose gel electrophoresis, transferred to GeneScreen Plus membrane and then hybridized to ^{32}P-labeled pODC54 probe. Lane 1, total RNA from N2a cells (16 μg); lane 2, total RNA from DF-40 cells (8 μg); lane 3, poly(A)$^+$ RNA from N2a cells (6 μg); lane 4, poly(A)$^+$ RNA from DF-40 cells (5 μg). The size of ODC mRNA was estimated by a comparison with the mobility of 28S and 18S ribosomal RNA. (C) SDS-polyacrylamide gel analysis of [^{3}H]DFMO affinity labeled ODC in N2a and DF-40 cells. Lane 1, N2a cells; lane 2, DF-40 cells. Confluent cultures of N2a and DF-40 cells were harvested and cytosolic extracts prepared for affinity labeling as described in Materials and Methods. Equal amounts of extracts (200 μg proteins) were processed for labeling and gel electrophoresis. Specific ODC activities for N2a and DF-40 cells in this particular experiment were, respectively, 1.1 units/mg protein and 243 units/mg proteins. The apparent molecular mass of labeled ODC band was determined by using the following molecular mass standards: phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

serum-deprivation were tested for their ability to induce the differentiation of DF-40 cells in culture. Fig. shows that both dibutyryl cAMP and DFMO/dibutyryl cAMP treatments induced extensive neurite outgrowth in N2a cells (Fig. 3A and B) but produced little or no morphological differentiation in DF-40 cells (Fig. 3C and D). No differentiation of DF-40 cells was observed even when higher concentrations of dibutyryl cAMP (2.5 mM) were used. In contrast, Fig. 4 shows that serum-deprivation was effective in inducing the differentiation of both N2a and DF-40 cells. Morphological differentiation was apparent for both cell types 2 days after serum-deprivation, and a extensive neurite network could be observed 4 days after serum-deprivation. We have isolated more than 10 clonal lines which are resistant to DFMO up to 40 mM and all showed similar behavior, indicating that the observed difference in response to various differentiation inducing agents is not a unique event for the particular clone that we examined. The morphological differentiation of DF-40 cells was also accompanied by a 4 to 6-fold increase in acetylcholinesterase activity in DF-40 cells (Table II).

Changes in polyamine content in DF-40 cells during differentiation

The total polyamine content (i.e., the sum of putrescine, spermidine and spermine) in the day 4 cultures of DF-40 cells treated with dibutyryl cAMP, DFMO/dibutyryl cAMP and serum-deprivation were, respectively, 31, 95 and 25 nmol/mg protein as compared to the value of 512 nmol/mg protein in the control cultures (Fig. 5). In the case of dibutyryl cAMP or DFMO/dibutyryl cAMP treatment, such a reduction apparently was not sufficient to bring about differentiation. It can be noted that the total polyamine content in the proliferating N2a mouse neuroblastoma cells ranges between 15 to 20 nmol/mg protein (Table I, also see Ref. 5). In the case of serum-deprivation, however, the remaining polyamine content in the

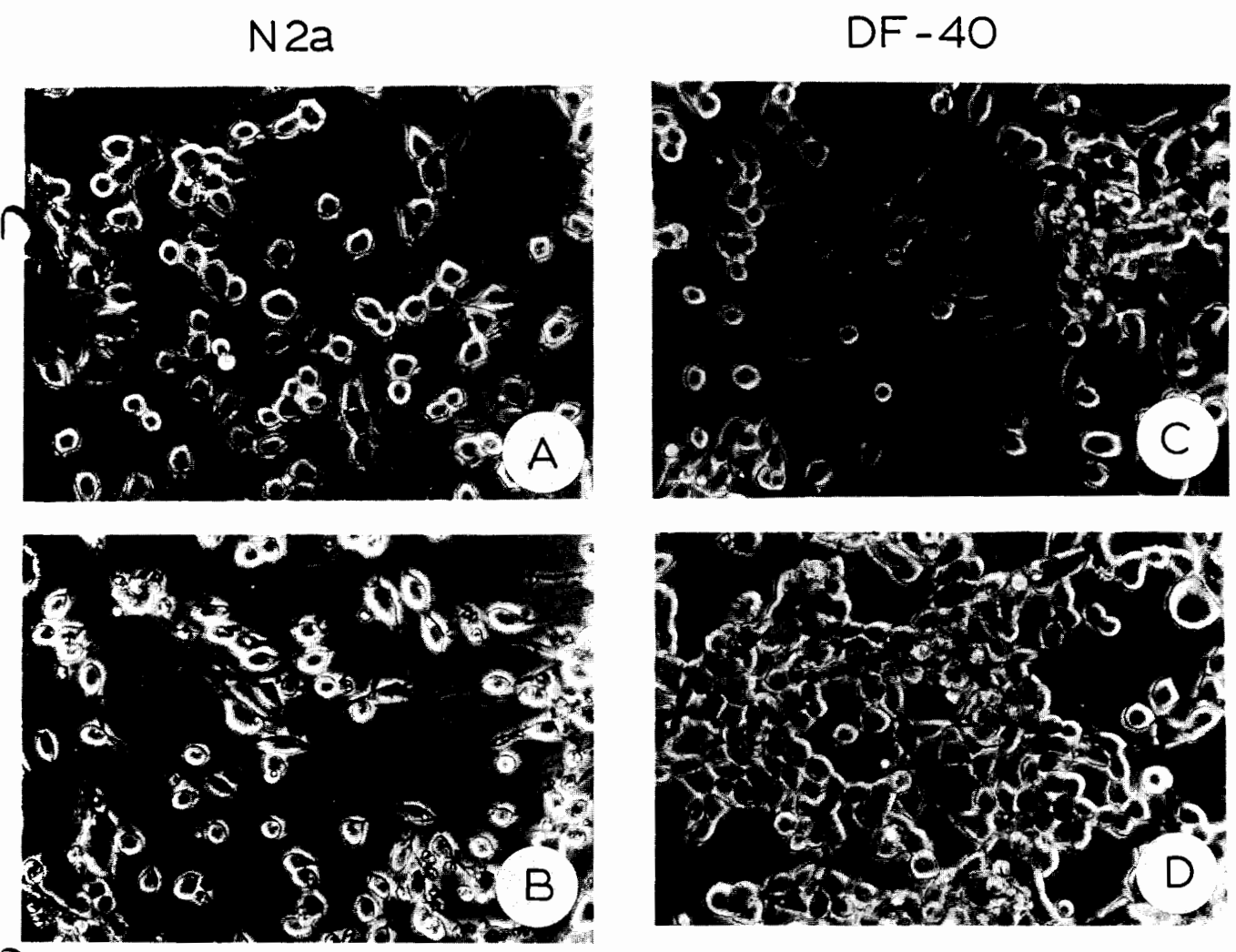

Fig. 3. Photomicrographs of N2a (A and B) and DF-40 (C and D) mouse neuroblastoma cells grown in the presence of 1 mM dibutyryl cAMP (A and C) and 1 mM DFMO plus 0.5 mM dibutyryl cAMP (B and D). Phase-contrast photomicrographs were taken 6 days after the initiation of the treatment.

TABLE II

Effect of serum-deprivation on acetylcholinesterase activity in DF-40 cells

	Day 2	Day 4	Day 6
Control [a]	6.5 [b]	7.7	
Serum-deprived [a]	6.5	33.8	25.4

[a] DF-40 cells were grown in the presence (control) or absence (serum-deprivation) of 10% fetal bovine serum. DF-40 cells in the control cultures reached confluence on day 4. More than 90% of DF-40 cells in the serum-deprived cultures exhibited neurite outgrowth on day 4. The morphology of the serum-deprived DF-40 cells remained differentiated in the day 6 cultures.

[b] Acetylcholinesterase activity was expressed in nmol of acetylcholine hydrolyzed per min per mg protein. Each value represent an average of triplicate measurements with errors less than 10% of the average.

treated cells, although still comparable to that in the undifferentiated N2a cells, was not able to prevent DF-40 cells from differentiation. Exogenously added putrescine, spermidine or spermine, up to 1 mM, could not block the differentiation of either DF-40 cells or N2a cells induced by serum-deprivation (data not shown).

Discussion

The ability of cAMP, or agents which increase the intracellular cAMP concentration, in inducing the differentiation of mouse neuroblastoma cells is well documented (reviewed in Ref. 24). Addition of 1 mM dibutyryl cAMP or other cAMP analogs to mouse neuroblastoma cells triggers the formation of neurites and the synthesis of neurospecific enzymes [24]. In previous studies, we have observed significant changes in polyamine metabolism, including a marked decrease in the ODC activity and the polyamine content [14], during the differentiation of mouse neuroblastoma cells, suggesting that the modulation of polyamine metabolism may be an integral part of the differentiation program. This notion is supported by the observation that specific inhibitors of ODC such as FMO and

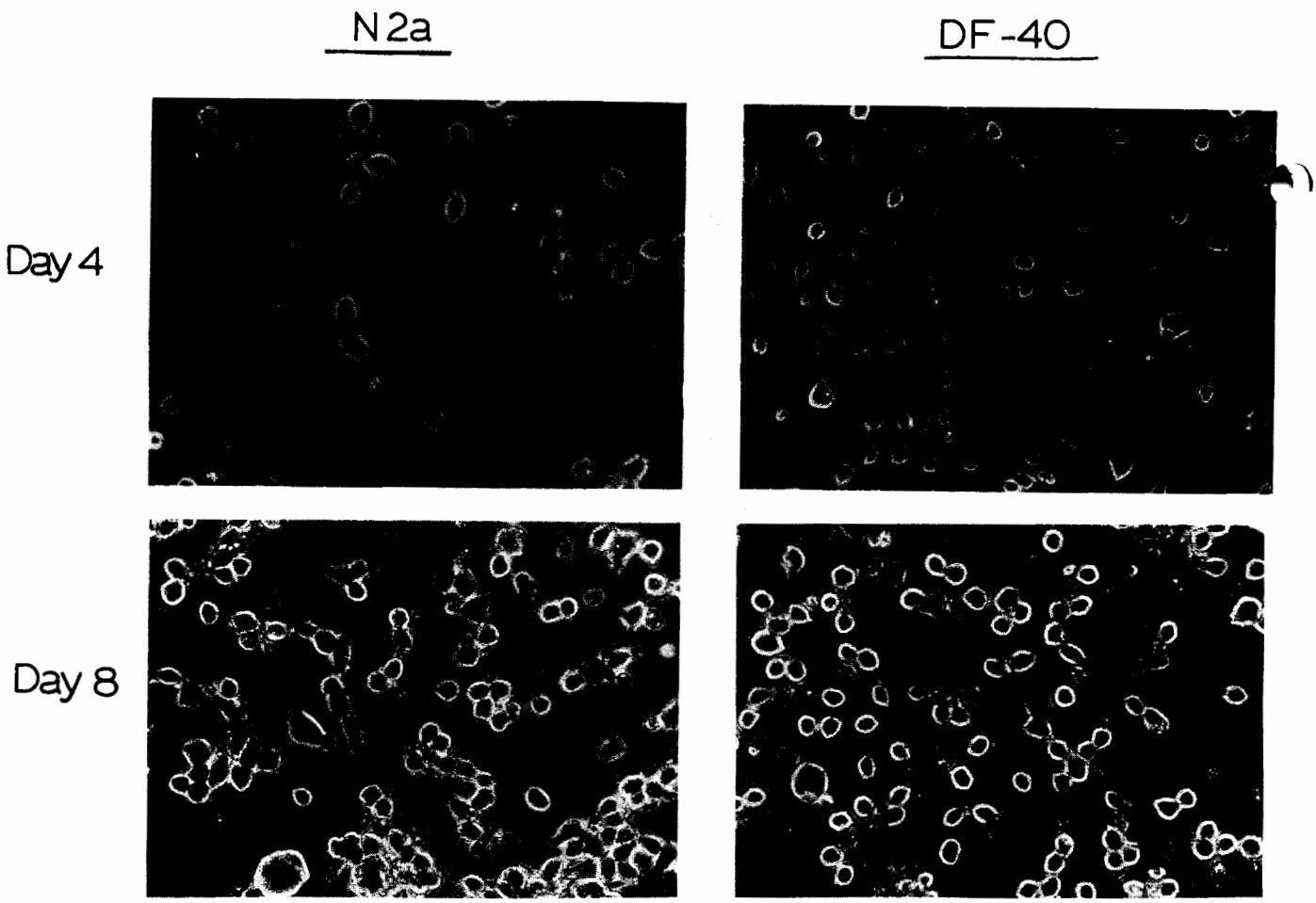

Fig. 4. Photomicrographs of N2a (left panels) and DF-40 (right panels) mouse neuroblastoma cells grown under serum-deprived condition. Serum-deprivation was initiated 10 h after plating cells in Dulbecco's growth medium containing 10% fetal bovine serum. Phase-contras photomicrographs of representative fields of the cell cultures were taken 4 and 8 days after the initiation of treatment. The diameter of the cell body was approx. 20 μm.

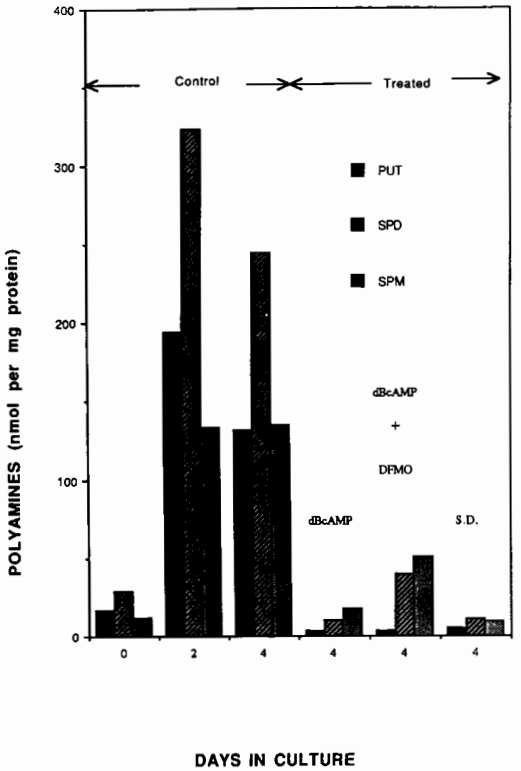

Fig. 5. Polyamine contents in DF-40 mouse neuroblastoma cells during normal growth (C. control) and 4 days after treatment with 1 mM dibutyryl cAMP (dBcAMP), 2 mM DFMO plus 0.3 mM dibutyryl cAMP (DFMO + dBcAMP) or serum-deprivation (S.D.). Cellular polyamine content was quantitated by HPLC as described in the text. The data presented were an average of two separate experiments with an error of less than 10%. PUT, putrescine; SPD, spermidine; SPM, spermine.

DFMO are effective in promoting mouse neuroblastoma differentiation [5]. A reduction in polyamine content by DFMO and other inhibitors in some tumor cell lines has also been shown to be effective in inducing the differentiated phenotypes in these cells [2–8].

In order to further understand the role of polyamines in regulating the differentiation of mouse neuroblastoma cells, we have attempted to generate ODC overproducers from N2a mouse neuroblastoma cells. We reasoned that if polyamines play a crucial role in tumor differentiation, DFMO-resistant ODC overproducers may be resistant to the action of differentiation-inducing agents. DFMO-resistant ODC overproducers have been generated from various mammalian cell lines [10–13,22]. Most of the studies have been focused on the use of these overproducers in obtaining ODC cDNA for studying ODC regulation, little attention has been paid to the physiology of these ODC overproducers. Recently, Pegg et al. [13] have studied the effect of *S*-adenosylmethionine decarboxylase inhibitors on the growth of DFMO-resistant L1210 cells. Using a similar selection protocol we have isolated many DFMO-resistant clones which can be grown even in the presence of 40 mM DFMO. Based on Southern blot analysis, we estimated that the DF-40 mouse neuroblastoma ODC overproducer contained about 40-fold more copies of the ODC gene (Fig. 2A). Levels of ODC mRNA and ODC molecules in this overproducer were significantly higher than in the parental N2a cells (Fig. 2B and C). When grown in the absence of DFMO, the intracellular polyamine content in DF-40 cells was two orders of magnitude higher than that in control N2a cells (Table I). Such high polyamine content did not appear to have an adverse effect on the morphology and growth of DF-40 cells.

Our studies showed that DF-40 cells were recalcitrant to the action of dibutyryl cAMP or DFMO/dibutyryl cAMP (Fig. 3) despite a more than 80 to 90% reduction of polyamine contents in treated DF-40 cells (Fig. 5). In contrast, both treatments were effective in eliciting differentiation of N2a cells, accompanied by a more than 80% reduction in polyamine content [5]. Although the degrees of reduction in the level of polyamines in both DF-40 and N2a cells were similar, the absolute amount of remaining polyamines in treated DF-40 cells was still comparable to or higher than that in proliferating N2a cells (Fig. 5). Taken together, these data would suggest that unless polyamine levels are brought down below a certain critical value (e.g., 15 nmol/mg protein), mouse neuroblastoma cells may not differentiate. This notion, however, contradicts with the observation that DF-40 cells could differentiate under the condition of serum-deprivation (Fig. 4) since the differentiated DF-40 cells still contained polyamine contents comparable to that in the undifferentiated N2a cells (25 nmol/mg protein vs. 20 nmol/mg protein). In addition, we found that exogenously added polyamines could not block the differentiation of the DF-40 cells induced by serum-deprivation. These results indicate that the level of polyamines alone, whether endogenous or exogenously added, could not block or reverse the differentiation of serum-deprived DF-40 cells. This is surprising since it is generally thought that differentiation of mouse neuroblastoma cells induced by serum-deprivation is mediated by cAMP [24,25]. Since the treatment of dibutyryl cAMP or DFMO/dibutyryl cAMP was carried out in the presence of serum, one possible explanation for the discrepancy is that the role of polyamines in modulating neuroblastoma differentiation may be serum dependent. This notion is also supported by the finding that exogenously added polyamines could not block the differentiation of N2a cells induced by serum-deprivation. It is possible that endogenous polyamines in serum-deprived DF-40 cells may be sequestered and hence are not available for growth regulation. Although little is known about the compartmentalization and trafficking of polyamines inside mammalian cells, active polyamine sequestration has been demonstrated

in *Neurospora crassa* [26]. Alternatively, it is possible that the growth-stimulatory action of polyamines needs the coordination of certain factors either present in serum or generated by serum through a signal transduction pathway within the cell. Thus, in the absence of serum or certain growth factors, polyamines cannot be utilized for growth stimulation or the blocking of differentiation. In summary, our study with the ODC overproducing DF-40 cell line suggested that the mechanism of the differentiation of mouse neuroblastoma cells induced by serum-deprivation may be different from that induced by dibutyryl cAMP or DFMO/dibutyryl cAMP, and such a difference may be related to the compartmentalization and/or serum-dependent utilization of cellular polyamines.

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