[CANCER RESEARCH 43, 2812–2818, June 1983]
0008-5472/83/0043-0000$02.00

Effects of Inhibitors of Ornithine Decarboxylase on the Differentiation of Mouse Neuroblastoma Cells

Kuang Yu Chen,[1] David Nau, and Alice Y-C. Liu[2]

Department of Chemistry, Rutgers-The State University of New Jersey, New Brunswick, New Jersey 08903 [K. Y. C., D. N.], and Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115 [A. Y-C. L.]

ABSTRACT

(*R*, *S*)-α-Fluoromethylornithine (α-FMO), a catalytic irreversible inhibitor of ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17), induced the differentiation of N2a mouse neuroblastoma cells. The effect of α-FMO was concentration dependent; approximately 50% of the cell population exhibited neurite outgrowth in the presence of 1 mM α-FMO, while higher concentrations caused severe growth inhibition and cell death. The effect of 1 mM α-FMO on neuroblastoma differentiation was potentiated greatly by 0.1 to 0.2 mM N^6,$O^{2'}$-dibutyryl adenosine cyclic 3′:5′-monophosphate (Bt_2cAMP) causing more than 90% of the cell population to differentiate morphologically with thick and long processes; 0.1 to 0.2 mM Bt_2cAMP, by itself, had no effect on cell growth and did not induce neurite outgrowth. The effect of α-FMO, either by itself or in combination with 0.1 to 0.2 mM Bt_2cAMP, on the morphological differentiation of mouse neuroblastoma cells was reversed by the addition of exogenous putrescine or spermidine. The morphological differentiation of mouse neuroblastoma cells induced by 1 mM α-FMO plus 0.2 mM Bt_2cAMP was accompanied by increases of the regulatory subunit of the type I cAMP-binding protein and acetylcholinesterase activity. These results indicate that the modulation of cellular polyamine contents may be important in neuroblastoma cell differentiation.

INTRODUCTION

The decarboxylation of L-ornithine by ODC[3] leads to the formation of putrescine and is the rate-controlling step in the biosynthesis of other polyamines, spermidine and spermine (1, 4, 25). Polyamines are ubiquitous organic cations in prokaryotic and eukaryotic cells and are implicated in many growth-regulatory processes (1, 9, 31). Although the involvement of ODC and polyamines in proliferative and neoplastic growth of mammalian cells is well documented, there have been relatively few studies on the possible roles of ODC/polyamines in cell differentiation. Rath and Reddi (29) demonstrated an increase of ODC activity during matrix-induced sequential differentiation of cartilage, bone, and bone marrow *in vivo*. Bethell and Pegg (2) reported an increase in spermidine content when confluent 3T3-L1 fibroblasts are stimulated to differentiate into adipocytes. On the other hand, Stoscheck *et al.* (35) observed a decrease in ODC activity of L6 myoblasts upon fusion to myotubes. Recently, we have found that the differentiation of mouse neuroblastoma cells in tissue culture, induced by 1 mM Bt_2cAMP plus 0.5 mM IBMX, is accompanied by an early inhibition of putrescine uptake (8) and marked decreases in cellular ODC activity and polyamine contents (7). These studies indicate that, in addition to their involvement in cell growth and proliferation, ODC/polyamines may have important roles in cell differentiation.

In attempting to delineate the function(s) of polyamines in the differentiation of mouse neuroblastoma cells, one possible approach is to either inhibit or augment the cellular polyamine contents and to observe the consequence of this effect. Recently, highly specific irreversible inhibitors of ODC, such as α-FMO and α-DFMO, have been developed (17, 23). Studies have shown that these compounds are potent and effective inhibitors of the enzyme, ODC, in various cultured cell systems (5, 20, 22, 27) and tissues (10–12, 21). The availability of these irreversible inhibitors enabled us to study the role of polyamine metabolism in the differentiation of mouse neuroblastoma cells. We report here that 1.0 mM α-FMO could induce the differentiation of N2a mouse neuroblastoma cells and that 0.1 to ~0.2 mM Bt_2cAMP further potentiated the effects of α-FMO on cell differentiation. Our findings provide a direct evidence that changes in polyamine metabolism may be an integral component in the differentiation of neuroblastoma cells.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium and fetal calf serum were obtained from Grand Island Biological Co., Grand Island, N. Y. Bt_2cAMP, putrescine, spermidine, spermine, aminoguanidine, and 5-dimethylamino-1-naphthalenesulfonyl chloride (dansyl chloride) were purchased from Sigma Chemical Co., St. Louis, Mo. L-[1-^{14}C]Ornithine (59 mCi/mmol) and [1-^{14}C]acetylcholine (26.5 mCi/mmol) were from Amersham Corp., Arlington, Ill. 8-N_3-[^{32}P]cAMP (50 to ~80 Ci/mmol) was from ICN Chemical Radioisotope Division, Irvine, Calif. α-FMO was kindly provided by Dr. J. Kollonitsch of Merck, Sharp & Dohme Research Laboratories, Rahway, N. J. α-DFMO was generously provided by the Merrell Research Center, Cincinnati, Ohio. High-pressure liquid chromatography grade solvents were purchased from Baker Chemical Co., Phillipsburg, N. J.

Cell Culture and Drug Treatment. N2a mouse neuroblastoma cells were grown as monolayer culture in Dulbecco's modified Eagle's medium (with 4500 mg glucose per liter) supplemented with 10% fetal calf serum. Cells were maintained at 37° in a water-jacketed Forma incubator (95% air and 5% CO_2). α-FMO (100 mM) and Bt_2cAMP (100 mM) solutions were prepared freshly in H_2O under sterile conditions before use and were added to cell culture immediately after subculture. The seeding cell density was approximately 1×10^4 cells/sq cm. Occasionally, drugs were added 5 to 10 hr after subculture. No difference in results was

[1] Recipient of Grant CA 24479 from the NIH. To whom requests for reprints should be addressed, at Department of Chemistry, Rutgers University, P. O. Box 939, Piscataway, N. J. 08854.

[2] Recipient of a research grant from the American Cancer Society, Massachusetts Chapter.

[3] The abbreviations used are: ODC, L-ornithine carboxy-lyase; Bt_2cAMP, N^6,$O^{2'}$-dibutyryl adenosine cyclic 3′:5′-monophosphate; IBMX, 3-isobutyl-1-methylxanthine; α-FMO, (*R*,*S*)-α-fluoromethylornithine; α-DFMO, DL-α-difluoromethylornithine; 8-N_3-[^{32}P]cAMP, 8-azidoadenosine cyclic 3′:5′-[^{32}P]monophosphate; cAMP, cyclic adenosine 3′:5′-monophosphate.

Received May 11, 1982; accepted February 28, 1983.

observed. Cell growth was measured by the number of viable cells attached to the growth surface. To study the effect of exogenous polyamines on neuroblastoma differentiation, putrescine or spermidine was added to the culture medium immediately after drug treatment. When spermidine was used, the culture medium also contained 0.05 mM aminoguanidine, a potent inhibitor of diamine oxidase (3), to prevent the formation of toxic oxidized products.

Biochemical Assays. ODC activity was determined, using a 12,000 × *g* supernatant obtained from cell homogenate, according to procedures described previously (4, 6). For the quantitation of putrescine, spermidine, and spermine, the method of Seiler and Wiechman (33) was adopted to prepare the dansyl derivatives of polyamines. The analysis of the dansylated polyamines was carried out by high-pressure liquid chromatography technique using a 7-μm octadecylsilane reverse-phase column. The solvent system used was acetonitrile/H_2O (85/15, v/v) as described elsewhere (7). Quantitation of the regulatory subunit of the type I cAMP-binding protein was carried out by a photoaffinity labeling technique with 8-N_3-[^{32}P]cAMP as described previously (18, 19). Briefly, neuroblastoma cell extracts were incubated with 1 μM 8-N_3-[^{32}P]cAMP (4 to 10 Ci/mmol) at 4° for 1 hr. The samples were then photolyzed and subjected to analysis by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and autoradiography to determine the amount of radioactivity incorporated into specific protein bands. Acetylcholinesterase activity present in neuroblastoma cell extracts was determined by a radiometric assay method according to procedures described previously (18, 19). The amount of [1-^{14}C]acetylcholine hydrolyzed was determined by liquid scintillation spectrometry. The results are expressed in nmol of acetylcholine hydrolyzed per min per mg protein at 37° with a substrate concentration of 1 × 10^{-4} M. Protein concentration was determined by a modified Lowry's method using bovine serum albumin as the standard (30).

RESULTS

In this study, both α-FMO and α-DFMO were used to study the effects of inhibition of ODC and depletion of cellular polyamines on neuroblastoma cell differentiation. The results obtained with α-DFMO were identical to those of α-FMO, and only those results obtained using α-FMO are presented.

Chart 1 shows the effect of α-FMO on the growth of N2a mouse neuroblastoma cells. A reduced rate of cell proliferation was observed with increasing concentrations of α-FMO. The growth-inhibitory effect of α-FMO on mouse neuroblastoma cells is similar to that of α-DFMO reported in other cell culture systems (5, 22).

In addition to the inhibitory effect of α-FMO on cell growth, we observed that a significant portion of the N2a mouse neuroblastoma cells in the α-FMO-treated culture became morphologically differentiated 5 to 6 days after initiation of the drug treatment. The extent of morphological differentiation, as characterized by extensive neurite outgrowth (>50 μm long), was dependent on the concentration of α-FMO used (Chart 2). The optimal concentration range of α-FMO for the induction of neurite outgrowth of the N2a cells was 0.5 to 1.5 mM. Under these conditions, about 50% of the cell population extended neurites. However, at higher concentrations of α-FMO (>3 mM), we observed not only growth inhibition but also an inhibition of neurite outgrowth; dead cells and cell debris were apparent in those cultures treated with high concentrations of α-FMO.

In an effort to search for a means to maximize the differentiation of N2a cells in the α-FMO-treated cell culture, we found that 0.1 to 0.2 mM Bt_2cAMP increased the percentage of neurite-bearing cells in the 1 mM α-FMO-treated culture from 50 to

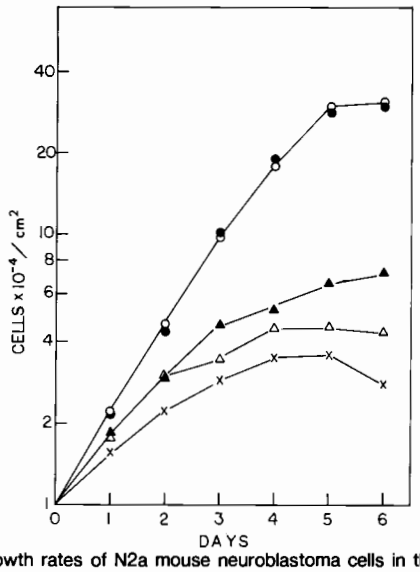

Chart 1. Growth rates of N2a mouse neuroblastoma cells in the presence of various concentrations of α-FMO and Bt_2cAMP. Mouse N2a neuroblastoma cells (1.8 × 10^5 cells/60-mm dish) were incubated from Time 0 in the absence (○) or presence of 0.2 mM Bt_2cAMP (●), 1 mM α-FMO (△), 1 mM α-FMO plus 0.2 mM Bt_2cAMP (▲), and 5 mM α-FMO (×). Cell number was determined by counting with a hemocytometer. *Points*, average of 3 dishes.

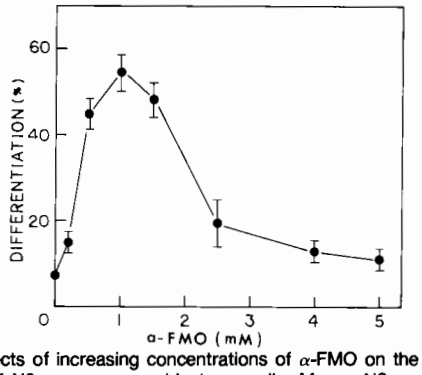

Chart 2. Effects of increasing concentrations of α-FMO on the morphological differentiation of N2a mouse neuroblastoma cells. Mouse N2a cells (1.8 × 10^5 cells/60-mm dish) were incubated with various concentrations of α-FMO from Time 0. Morphologically differentiated cells (cells with processes that were >50 μm long) were scored 6 days after initiation of the drug treatment. At least 300 cells were counted, and the number of differentiated cells was expressed as a percentage of total viable cells. *Points*, means of at least 3 determinations. *Bars*, S.D.

~90% (Chart 3). It should be noted, however, that 0.1 to 0.2 mM Bt_2cAMP, by itself, neither affects cell growth (Chart 1) nor induces morphological differentiation (Chart 3). Furthermore, the percentage of differentiated cells achieved in the presence of 1 mM α-FMO plus 1 mM Bt_2cAMP did not exceed that achieved in the presence of 1 mM α-FMO plus 0.1 to 0.2 mM Bt_2cAMP. Fig. 1 shows the photomicrographs of N2a mouse neuroblastoma cells grown in the presence of 0.2 mM Bt_2cAMP alone (Fig. 1*A*) and cells grown in the presence of a combination of α-FMO and Bt_2cAMP at various concentrations (Fig. 1, *B* to *D*). It can be seen that cells grown in the presence of 0.5 to 1.5 mM α-FMO plus 0.1 to 0.2 mM Bt_2cAMP exhibited thick and long processes, sometimes more than 600 μm long, forming extensive neurite webs. In contrast, cells grown in the presence of 0.2 mM Bt_2cAMP were morphologically identical to those of the control; in both cases, cells appeared as rounded and formed clumps upon reaching a stationary phase of growth (Fig. 1*A*). Morphologically differentiated mouse neuroblastoma cells induced by 1

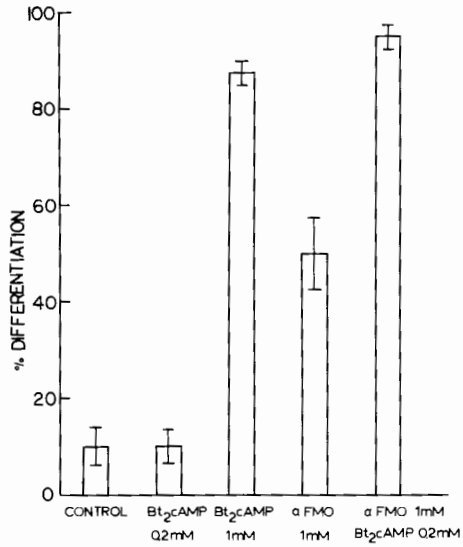

Chart 3. Effects of various concentrations of α-FMO and Bt_2cAMP, either alone or in combination, on the morphological differentiation of N2a mouse neuroblastoma cells. Cells were plated at a low density (1.6×10^5 cells/60-mm dish) in the presence of various drugs as indicated. Morphologically differentiated cells (cells with neurites >50 μm long) were scored on Day 7. At least 300 cells were counted, and the number of differentiated cells was expressed as a percentage of total viable cells (*i.e.*, trypan blue negative).

mM α-FMO (with or without 0.2 mM Bt_2cAMP) gave an approximately 2-fold increase of both regulatory subunit of the type I cAMP-binding protein (M_r 47,000) and acetylcholinesterase activity over those of the untreated control (Table 1). Both of these parameters have been used as indices for the biochemical differentiation of mouse neuroblastoma cells (7, 18, 19).

The effects of α-FMO, either alone or in combination with 0.1 to 0.2 mM Bt_2cAMP, on neuroblastoma cell differentiation appeared to be mediated through alteration of polyamine metabolism as suggested by results of the following experiments. Charts 4 and 5 illustrate the effects of 1 mM α-FMO and 0.2 mM Bt_2cAMP (either alone or in combination) on cellular ODC activity and individual polyamine contents, respectively, in the N2a neuroblastoma cells. The inhibitory effect of 1 mM α-FMO on cellular ODC activity was evident as early as 1 day after its addition (Chart 4). The time course of depletion of putrescine content paralleled that of the decrease in ODC activity, approaching a barely detectable level (0.05 pmol/mg protein) after Day 1 (Chart 5*A*). Qualitatively similar results on the effects of α-DFMO on ODC activity and putrescine content in other cell lines have been reported (5, 21, 25).

The characteristic increases of spermidine and spermine levels associated with logarithmic growth of the N2a mouse neuroblastoma cells were inhibited by the addition of 1 mM α-FMO (Chart 5, *B* and *C*). Cellular concentrations of spermidine and spermine remained at basal levels throughout the 7-day incubation period in the α-FMO-treated cultures. Identical results were obtained with α-DFMO (data not shown). It is interesting to note that Mamont *et al.* (22) found depletion of putrescine and spermidine but not spermine in HTC hepatoma cells and in mouse leukemia cells treated with α-DFMO. The discrepancy between their findings and ours may be due to different cell systems used. Indeed, we noticed that, under their experimental conditions, there is no growth-associated increase of spermine content in both of their control cultures.

It is noteworthy that the time course of changes in ODC activity and polyamine contents in the control N2a cell culture was similar to that described for the NB-15 mouse neuroblastoma cells reported previously (7). In addition, 0.1 to ~0.2 mM Bt_2cAMP had no effect on ODC activity or polyamine contents either by itself or when added together with 1 mM α-FMO. This result suggests that the potentiating effect of suboptimal concentrations of Bt_2cAMP on the α-FMO-induced neuroblastoma differentiation may not be related directly to effects of Bt_2cAMP on ODC/polyamines.

To further examine the role of ODC and polyamines in mouse neuroblastoma differentiation, we investigated whether exogenous polyamines would antagonize the action of α-FMO and Bt_2cAMP in neuroblastoma cell differentiation. Fig. 2 shows that putrescine or spermidine could reverse the differentiation of N2a cells induced by 1 mM α-FMO plus 0.1 mM Bt_2cAMP. Similar results were obtained when 1 mM α-FMO was used by itself to induce neuroblastoma cell differentiation (data not shown). When spermidine was used to study its effect on the α-FMO-induced neuroblastoma cell differentiation, the presence of aminoguanidine was necessary to inhibit the diamine oxidase activity present in the fetal calf serum-containing growth medium. In the absence

Table 1

Effects of α-FMO treatment on the regulatory subunit of the type I cAMP-binding and acetylcholinesterase activities of N2a mouse neuroblastoma cells

N2a mouse neuroblastoma cells were harvested at an early stationary phase of cell growth. The cell extracts (both cell homogenates and cytosols) were dialyzed extensively against phosphate buffer (pH 7.2, 50 mM) prior to assaying for regulatory subunit of the type I cAMP-binding and acetylcholinesterase activities. All assays were done under standard conditions according to methods described in the text.

	Regulatory subunit of the type I cAMP binding activities (pmol/mg protein)		Acetylcholinesterase (nmol/min/mg protein)	
	Cell homogenate	Cytosol	Cell homogenate	Cytosol
Control	1.8	3.8	13.6	30
α-FMO (1 mM)	3.4	7.4	24.2	61
α-FMO (1 mM) + Bt_2cAMP (0.2 mM)	4.3	6.9	30.6	52

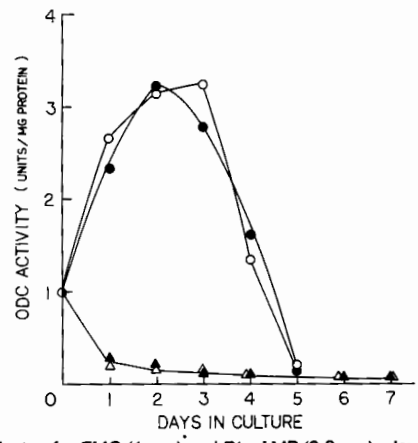

Chart 4. Effects of α-FMO (1 mM) and Bt_2cAMP (0.2 mM), alone or in combination, on cellular ODC activity as a function of time after their addition to the N2a cells. N2a mouse neuroblastoma cells were subcultured (seeding density, 1.6×10^5 cells/60-mm dish) on Day 0 in the absence (○) or presence of 0.2 mM Bt_2cAMP (●), 1 mM α-FMO (△), and 1 mM α-FMO plus 0.2 mM Bt_2cAMP (▲). ODC activity was determined as described in "Materials and Methods." Results are the average of 2 separate experiments.

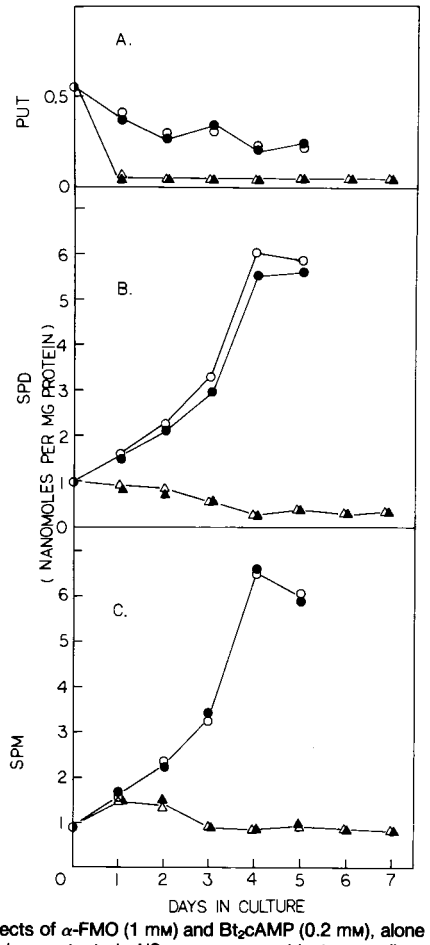

Chart 5. Effects of α-FMO (1 mM) and Bt_2cAMP (0.2 mM), alone or in combination, on polyamine contents in N2a mouse neuroblastoma cells as a function of time in culture. Cells were subcultured (seeding density, 1.6×10^5 cells/60-mm dish) on Day 0 in the absence (○) or presence (●) or 0.2 mM Bt_2cAMP, 1 mM α-FMO (△), and 1 mM α-FMO plus 0.2 mM Bt_2cAMP (▲). Polyamines were quantitated by the high-pressure liquid chromatography method described in the text. *PUT*, putrescine; *SPD*, spermidine; *SPM*, spermine.

of aminoguanidine, exogenously added spermidine was extremely toxic to the N2a cells; this may be attributable to the formation of oxidized products from spermidine under these conditions. Interestingly, exogenous putrescine can be added directly to the fetal calf serum-containing medium without any adverse effects on the growth of neuroblastoma cells. The reversion effect of putrescine on the α-FMO-induced neuroblastoma differentiation was not affected by the presence of aminoguanidine.

The results that exogenous polyamines antagonize the action of α-FMO on N2a neuroblastoma cell differentiation provide strong evidence that the decrease of cellular polyamines plays an important role in the differentiation of mouse neuroblastoma cells.

Interestingly enough, exogenous putrescine did not reverse the differentiation of mouse neuroblastoma cells induced by 1 mM Bt_2cAMP or 1 mM Bt_2cAMP plus 0.5 mM IBMX (data not shown). This could be due to a decreased putrescine uptake in the cAMP-treated cells as we have reported previously (8).

DISCUSSION

Mouse neuroblastoma cells in tissue culture can be induced to differentiate by the addition of cAMP analogs or agents which increase intracellular cAMP concentration (12, 28). Such differentiation is characterized by the morphological appearance of neurite outgrowth and the biochemical appearance of enzyme activities related to neurotransmitter metabolism. The molecular mechanisms of cAMP-induced neuroblastoma differentiation may have a broad implication, because cAMP is known to be involved in the differentiation of a variety of animal cells including melanoma (15, 24), pheochromocytoma (14, 32), granulosa (16), and muscle cells (35). On the basis of the observations that the differentiation of mouse neuroblastoma cells induced by 1 mM Bt_2cAMP and 0.5 mM IBMX (a phosphodiesterase inhibitor) is accompanied by a 10-fold increase in the apparent K_m value of putrescine transport (8) and a 5- and 15-fold decrease, respectively, of spermidine and cellular ODC activity (7), we have proposed that one of the primary target sites of cAMP action in neuroblastoma differentiation may be the alteration of polyamine metabolism (7). Indeed, many studies using cells in tissue culture and experimental animals have suggested that cAMP can modulate cellular ODC activity (for reviews, see Refs. 4 and 31).

The present study is aimed at further elucidating the role of ODC and polyamines in mouse neuroblastoma differentiation. Our results suggest that: (*a*) α-FMO, at optimal concentrations (1 to 1.5 mM), can induce the morphological differentiation of mouse N2a neuroblastoma cells; (*b*) the action of α-FMO on neuroblastoma differentiation appears to be mediated through inhibition of ODC activity and reduction of polyamine contents; (*c*) suboptimal concentrations of Bt_2cAMP (0.1 to 0.2 mM) can potentiate the effect of α-FMO on mouse neuroblastoma differentiation; such a potentiating effect does not appear to be related to further changes of ODC activity and polyamine contents.

The apparently bell-shaped dose-response curve (Chart 2) of the effect of α-FMO on neuroblastoma cell differentiation may be related to toxicity of high concentrations of α-FMO (>3 mM), since floating cells and cell debris appeared in these cultures 4 to 5 days after initiation of the treatment. Alternatively, the phenomena observed with high concentrations of α-FMO (inhibition of cell differentiation, cell growth, and ultimately cell death) may be related to an observation made recently by Pohjanpelto *et al.* (26). They reported that polyamine starvation causes disappearance of microtubules and actin filaments in polyamine-auxotrophic Chinese hamster ovary cells. It is possible that α-FMO at higher concentrations may severely deplete the cellular polyamine contents to the extent that neurite formation as well as cell division become impossible.

The observation that exogenous putrescine or spermidine can block the action of 1 mM α-FMO, either alone or with 0.1 to 0.2 mM Bt_2cAMP, on neuroblastoma cell differentiation (Fig. 2) suggests that modulation of ODC activity and polyamine contents may bear a causal relationship to neuroblastoma cell differentiation. Since the extensive neurite webs became prominent approximately 5 days after the addition of α-FMO, whereas the inhibition of ODC activity and polyamine contents was apparent on Day 1, its seems likely that the alteration of ODC/polyamine may trigger a chain of biochemical events that, after a lag period, may lead to cell differentiation.

The mechanism through which suboptimal concentrations of Bt_2cAMP potentiate the effect of α-FMO on neuroblastoma cell differentiation is not clear. Gunning *et al.* (14) have reported a synergistic effect of Bt_2cAMP and β-nerve growth factor on the

neurite outgrowth of PC-12 pheochromocytoma cells and suggested that Bt_2cAMP is responsible for the initiation of neurite outgrowth. In light of their study, the potentiating role of Bt_2cAMP on the α-FMO-induced neuroblastoma differentiation may be related to microtubule assembly. The possible involvement of cAMP and protein phosphorylation in microtubule polymerization has been proposed (34, 36). Alternatively, it is possible that low concentrations of Bt_2cAMP may modify a certain biochemical event(s) which by itself has no effect on tumor growth or differentiation but can "prime" or prepare cells for maximal differentiation when cellular ODC activity and polyamine contents are reduced by α-FMO treatment.

Tumor differentiation is undoubtedly a complex phenomenon. Bethell and Pegg (2) have shown that α-DFMO inhibits the differentiation of 3T3-L1 preadipocytes, suggesting the involvement of ODC/polyamines in normal cell differentiation. Similarly, Fozard *et al.* (11, 12) have demonstrated the need of ODC-polyamines for embryonic development. On the other hand, Luk *et al.* (20) have found that α-DFMO inhibits cell growth but has no effect on the chemically induced differentiation of promyelocytic HL-60 cells, implying that polyamines are only involved in the proliferation but not in the terminal differentiation of these cells.

The relationship between the proliferation and differentiation of mouse neuroblastoma cells is complex as indicated by the following observations: (*a*) the differentiation of mouse neuroblastoma cells is associated generally with a decrease of cellular growth rate (7, 19, 28). (*b*) inhibition of cell growth does not necessarily induce neuroblastoma cell differentiation (18); (*c*) maximal expression of differentiated phenotypes of mouse neuroblastoma cells requires several cycles of cell division after the addition of 1 mM Bt_2cAMP to the cell culture (7, 19).

The present data together with our previous findings that neuroblastoma differentiation is accompanied by a decrease of ODC/polyamines (7) suggest that the modulation of ODC/polyamines may be important in the differentiation of mouse neuroblastoma cells; however, the precise roles of ODC/polyamines in neuroblastoma differentiation and proliferation remain to be elucidated.

REFERENCES

1. Bachrach, U. Function of Naturally Occurring Polyamines, pp. 1–211. New York: Academic Press, Inc., 1973.
2. Bethell, D. R., and Pegg, A. E. Polyamines are needed for the differentiation of 3T3-L1 fibroblasts into adipose cells. Biochem. Biophys. Res. Commun., *102:* 272–278, 1981.
3. Burkard, W. P., Gey, K. F., and Pletscher, A. Inhibition of diamine oxidase *in vivo* by hydrazine derivatives. Biochem. Pharmacol., *3:* 249–255, 1960.
4. Canellakis, E. S., Viceps-Madore, D., Kyriakidis, D. A., and Heller, J. S. The regulation and function of ornithine decarboxylase and of polyamines. Curr. Top. Cell. Regul. *15:* 156–202, 1979.
5. Chapman, S. K. Antitumor effects of vitamin A and inhibitors of ornithine decarboxylase in cultured neuroblastoma and glioma cells. Life Sci., *26:* 1359–1366, 1980.
6. Chen, K. Y., Heller, J. S., and Canellakis, E. S. Studies on the regulation of ornithine decarboxylase activity by the microtubules. The effect of colchicine and vinblastine. Biochem. Biophys. Res. Commun., *68:* 401–408, 1976.
7. Chen, K. Y., Presepe, V., Parken, N., and Liu, A. Y-C. Changes of ornithine decarboxylase activity and polyamine content upon differentiation of mouse NB-15 neuroblastoma cells. J. Cell Physiol., *110:* 285–290, 1982.
8. Chen, K. Y., and Rinehart, C. A., Jr. Difference in putrescine transport in undifferentiated *versus* differentiated mouse NB-15 neuroblastoma cells. Biochem. Biophys. Res. Commun., *101:* 243–249, 1981.
9. Cohen, S. S. Introduction to Polyamines, pp. 1–179. Englewood Cliffs, N. J.: Prentice-Hall, Inc., 1971.
10. Danzin, C., Jung, J. J., Grove, J., and Bey, P. Effect of α-difluoromethylornithine, an enzyme-activated irreversible inhibitor of ornithine decarboxylase, on polyamine levels in rat tissues. Life Sci., *24:* 519–524, 1979.
11. Fozard, J. R., Part, M., Prakash, N. J., and Grove, J. Inhibition of murine embryonic development by α-difluoromethylornithine, an inhibitor of ornithine decarboxylase. Eur. J. Pharmacol., *65:* 379–391, 1980.
12. Fozard, J. R., Part, M., Prakash, N. J., Grove, J., Schechter, P. J., Sjoerdsma, A., and Koch-Weser, J. L-ornithine decarboxylase: an essential role in early mammalian embryogenesis. Science (Wash. D. C.), *208:* 505–508, 1980.
13. Furmanski, P., Silverman, D. J., and Lubin, M. Expression of differentiated functions in mouse neuroblastoma mediated by dibutyryl cyclic adenosine monophosphate. Nature (Lond.), *233:* 413–415, 1971.
14. Gunning, P. W., Landreth, G. E., Bothwell, M. A., and Shotter, E. M. Differential and synergistic actions of nerve growth factor and cyclic AMP in PC 12 cells. J. Cell Biol., *89:* 240–246, 1981.
15. Johnson, G. S., and Pastan, I. N^6,$O^{2'}$-Dibutyryl adenosine 3′:5′-monophosphate induces pigment production in melanoma cells. Nature (Lond.), *237:* 267–268, 1972.
16. Knecht, M., Amsterdam, A., and Catt, K. The regulatory role of cyclic AMP in hormone-induced granulosa cell differentiation. J. Biol. Chem., *256:* 10,628–10,633, 1981.
17. Kollonitsch, J., Patchett, A. A., Marburg, S., Maycock, A. L., Perkins, L. M., Doldouras, G. A., Duggan, D. E., and Aster, S. D. Selective inhibitors of biosynthesis of aminergic neurotransmitters. Nature (Lond.), *274:* 906–908, 1978.
18. Liu, A. Y-C., Chan, T., and Chen, K. Y. Induction of the regulatory subunit of type 1 adenosine cyclic 3′:5′-monophosphate-dependent protein kinase in differentiated N-18 mouse neuroblastoma cells. Cancer Res., *41:* 4579–4587.
19. Liu, A. Y-C., Fiske, W. W., and Chen, K. Y. Regulation cyclic adenosine 3′:5′-monophosphate-binding protein in N-18 mouse neuroblastoma cells. Cancer Res., *40:* 4100–4108, 1980.
20. Luk, G. D., Civin, C. I., Weissman, R. M., and Baylin, S. B. Ornithine decarboxylase: essential in proliferation but not differentiation of human promyelocytic leukemia cells. Science (Wash. D. C.), *216:* 75–77, 1982.
21. Luk, G. D., Marton, L. J., and Baylin, S. B. Ornithine decarboxylase is important in intestinal mucosal maturation and recovery from injury in rats. Science (Wash. D. C.), *210:* 195–198, 1980.
22. Mamont, P. S., Duchesne, M. C., Grove, J., and Bey, P. Antiproliferative properties of D,L-α-difluoromethylornithine in cultured cells. A consequence of the irreversible inhibition of ornithine decarboxylase. Biochem. Biophys. Res. Commun., *81:* 58–66, 1978.
23. Metcalf, B. W., Bey, P., Danzin, C., Jung, J. J., Casara, P., and Vevert, J. P. Catalytic irreversible inhibition of mammalian ornithine decarboxylase by substrate and product analogs. J. Am. Chem. Soc., *100:* 2551–2553, 1978.
24. Pawelek, J. M. Factors regulating growth and pigmentation of melanoma cells. J. Invest. Dermatol., *66:* 201–209, 1976.
25. Pegg, A. E., and Williams-Ashman, H. G. Biosynthesis of putrescine in the prostate gland of the rat. Biochem. J., *108:* 533–539, 1968.
26. Pohjanpelto, P., Virtanen, I., and Holtta, E. Polyamine starvation causes disappearance of actin filaments and microtubules in polyamine-auxotrophic CHO cells. Nature (Lond.), *293:* 475–477, 1981.
27. Prakash, J. J., Schechter, P. J., Mamont, P. S., Grove, J., Koch-Weser, J., and Sjoerdsma, A. Inhibition of EMT-6 tumor growth by interference with polyamine biosynthesis: effect of α-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase. Life Sci., *26:* 181–194, 1980.
28. Prasad, K. N., and Hsie, A. W. Morphological differentiation of mouse neuroblastoma cells induced *in vitro* by dibutyryl adenosine 3′:5′-cyclic monophosphate. Nat. New Biol., *233:* 141–142, 1971.
29. Rath, N. C., and Reddi, A. H. Changes in ornithine decarboxylase activity during matrix-induced cartilage, bone and bone marrow differentiation. Biochem. Biophys. Res. Commun., *81:* 106–113, 1978.
30. Ross, E., and Shatz, G. Assay of protein in the presence of high concentrations of sulfhydryl compounds. Anal. Biochem., *54:* 304–306, 1973.
31. Russell, D. H., and Durie, B. G. M. Polyamines as biochemical markers of normal and malignant growth, pp. 1–178. New York: Raven Press, 1978.
32. Schubert, D. S., and Whitlock, C. Alteration of cellular adhesion by nerve growth factor. Proc. Natl. Acad. Sci. U. S. A., *74:* 4055–4058, 1977.
33. Seiler, N., and Wiechman, M. TLC analysis of amines as their DANS-derivatives. *In:* A. Niederwieser and G. Patake (eds.), Progress in Thin-Layer Chromatography and Related Methods, Vol. 1, pp. 94–145. Ann Arbor, Michigan: Humphrey Science Publishers, 1970.
34. Sloboda, R. D., Rudolph, S. A., Rosenbaum, J. L., and Greengard, P. Cyclic AMP-dependent endogenous phosphorylation of a microtubule associated protein. Proc. Natl. Acad. Sci. U. S. A., *72:* 177–181, 1975.
35. Stoscheck, C. M., Florini, J. R., and Richman, R. A. The relationship of ornithine decarboxylase activity to proliferation and differentiation of L6 muscle cells. J. Cell Physiol., *102:* 11–18, 1980.
36. Tash, J. S., Lagace, L., Lynch, D. R., Cox, S. M., Brinkley, B. R., and Means, A. R. Role of cAMP-dependent protein phosphorylation in microtubule assembly and function. Cold Spring Harbor Conf. Cell Proliferation, *8:* 1171–1185, 1981.

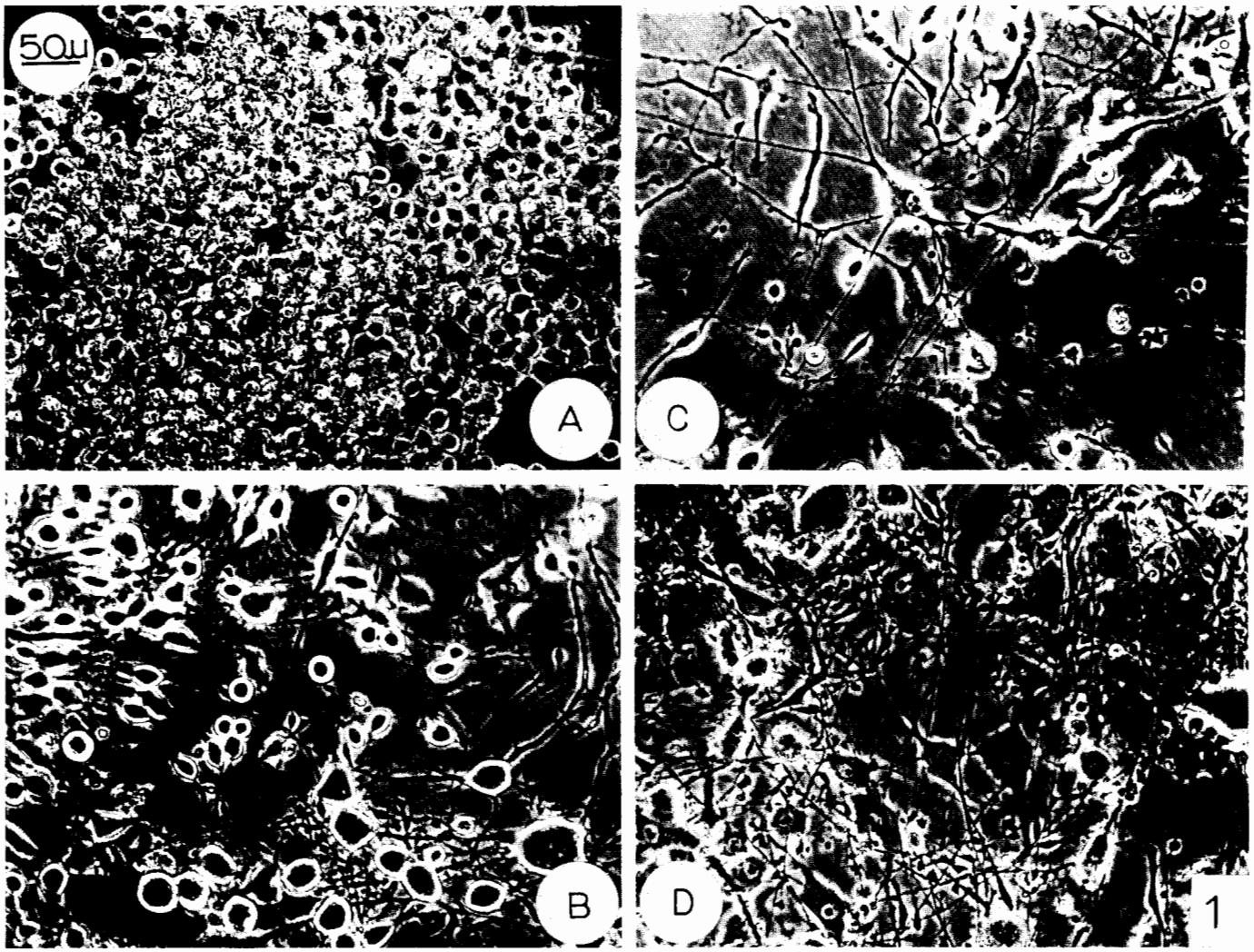

Fig. 1. Photomicrographs of N2a mouse neuroblastoma cells (unstained) treated with 0.2 mM Bt_2cAMP (*A*), 1 mM α-FMO plus 0.2 mM Bt_2cAMP (*B*), 1.5 mM α-FMO plus 0.1 mM Bt_2cAMP (*C*), and 0.5 mM α-FMO plus 0.2 mM Bt_2cAMP (*D*). Phase-contrast photomicrographs of representative fields of the cell cultures were taken 8 days after initiation of the treatment. *Bar at upper left*, 50 μm.

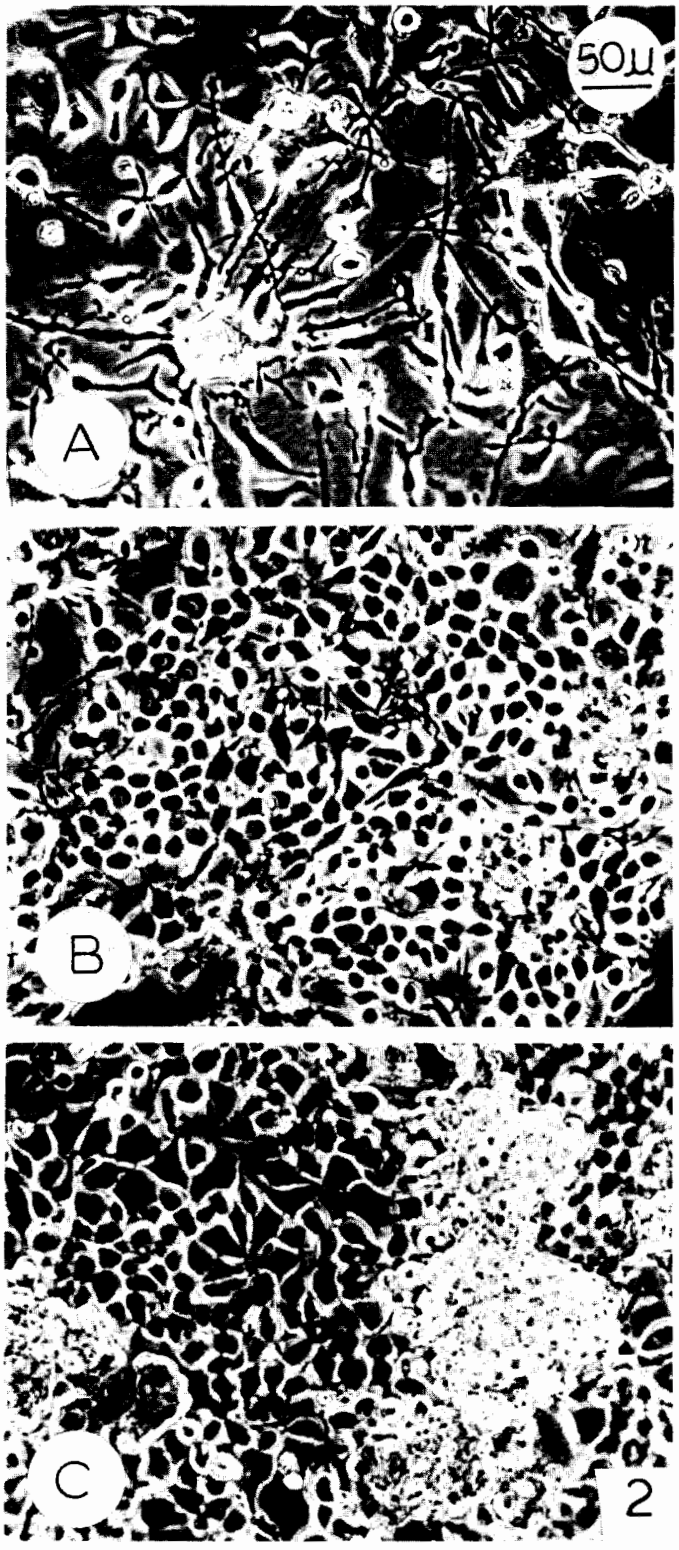

Fig. 2. Effects of exogenous polyamine in antagonizing the action of α-FMO and Bt_2cAMP in neuroblastoma cell differentiation. N2a mouse neuroblastoma cells were plated at a density of 1.2×10^5 cells/60-mm dish; 1 mM α-FMO and 0.2 mM Bt_2cAMP were added at the time of seeding ($t = 0$) to induce cell differentiation. When used, exogenous polyamines were added at the same time. *A*, control; *B*, 0.1 mM putrescine added; *C*, 0.1 mM spermidine and 50 μM aminoguanidine added. Phase-contrast photomicrographs of representative fields of the cell cultures (*unstained*) were taken on Days 7, 5, and 5 for *A*, *B*, and *C*, respectively. *Bar* at *upper left*, 50 μm.