Human Neuroblastoma Cell Culture: Effect of 5-Bromodeoxyuridine on Morphological Differentiation and Levels of Neural Enzymes (37522)

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5-Bromodeoxyuridine (5-BrdU), an analog of thymidine, has contrasting effects on the expression of differentiated phenotype and its associated functions in mammalian cell culture. For example, 5-BrdU suppresses the appearance of certain differentiated functions in embryonic tissue and mammary gland *in vitro* (1–5), and reduces the tumorigenecity of mouse melanoma cells in culture (6). This agent causes a rapid inhibition of the synthesis of adrenal-steroid-inducible tyrosine aminotransferase and slower decrease in the concentrations of lactate dehydrogenase, alcohol dehydrogenase and glucose-6-phosphate dehydrogenase; however, it does not affect the levels of malate dehydrogenase, acid phosphatase or alanine aminotransferase (7). 5-BrdU has an opposite effect on mouse neuroblastoma cell culture. It induces morphological differentiation (8) and increases the level of choline acetyltransferase (ChA), catechol-*o*-methyl-transferase (COMT) and tyrosine hydroxylase (9). We report now that 5-BrdU causes "differentiation" of human neuroblastoma cells in culture as evidenced by the formation of long neurites and by the induction of tyrosine hydroxylase (TH) and catechol-*o*-methyl-transferase (COMT). The level of cyclic AMP in 5-BrdU-treated cells does not change.

Materials and Methods. Human neuroblastoma culture (IMR-32 clone) was obtained from American Tissue Culture Association. These cells are grown in Falcon plastic flasks or dishes containing Eagle's medium and 10% heat inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml), and are maintained at 36° in a humidified atmosphere of 5% CO_2 in air. These cells grow in clumps and cell to cell adhesion appears to be firmer than that of mouse neuroblastoma cells. The attachment efficiency after replating is poor and cells attach to flask surface more loosely than mouse neuroblastoma cells. The doubling time of human neuroblastoma cells is about 48 hr. Unlike mouse neuroblastoma cells, these cells are not round-shaped. The spontaneous morphological differentiation (cytoplasmic processes greater than 50 μm in length) is less than 1% of total cells. This clone of human neuroblastoma tumor has ChA (10). For the study of morphological differentiation cells were plated in the Falcon dishes (60 mm) and 5-BrdU (0.5, 1, 2.5, and 5 μM), was was added 4 days after plating. This time interval allows maximal attachment and each dish has an adequate number of exponentially growing cells. One set of control received an equivalent volume of solvent, while other set of control received no treatment. The drug and medium were changed every 2 days. The culture was observed for a period of 10–13 days after treatment. The attachment efficiency varies from one dish to another. The clumping of cells is severe during growth. In addition, many cellular clumps come off the dish surface and this event varies from one dish to another. Because of the above problems, it was impossible to make a quantitative estimation at this time. Therefore, the data were presented in the form of photomicrographs. For enzyme analysis, cells were plated in large Falcon plastic flask (75 cm^2) and 5-BrdU (0.5 μM) was added 2 days later. Enzymes were assayed 9–10 days after treatment. Fresh medium and drug were replaced 5 and 8 days after treatment. Cells

were removed using 0.25% Viokase solution and equal volume of medium was added. The cells were washed once with medium and twice with PDS buffer. The TH activity was measured according to the method of Waymire *et al.* (11). The procedure involves the recovery and assay of $^{14}CO_2$ after decarboxylation with partially purified hog aromatic L-amino acid decarboxylase of carboxy-labeled dihydroxyphenylalanine formed from carboxyl labeled tyrosine. The ChA was measured according to the method of Fellman (12) as modified by Waymire *et al.* (unpublished observation). The procedure involves conversion of choline to acetylcholine by ChA in the presence of ^{14}C-acetylcoenzyme A. The COMT was assayed according to the method of Axelrod *et al.* (13). This method involves conversion of norepinephrine to ^{14}C-metanorepinephrine by COMT in the presence of S-adenosyl-L-methionine (^{14}C-methyl). The cyclic AMP level was measured according to Gilman's method (14) and the protein was determined according to the method of Lowry *et al.* (15).

Results and Discussion. 5-BrdU induced morphological differentiation in human neuroblastoma cell culture which is concentration and time dependent. The optimal concentration appeared to be about 2.5 μM and optimal time about 9–10 days after treatment. Figures 2B and C show the extent of "differentiation" in densely and sparsely populated areas of neuroblastoma cell culture, respectively. Many cells did not form neurites. Because of severe clumping it was impossible to quantitate the extent of differentiation. Gross observation at 10 days after treatment indicates that cells continued to grow in the presence of drug, but at a higher concentration (5 μM), the growth rate appeared to be slower than that of controls. On removal of the drug 10 days after treatment, many cells with neurites were observed 6 days later. Some dead cells were floating in the medium indicating that "differentiated" cells as a function of time may die in culture. The addition of an equimolar of thymidine together with 5-BrdU did not reduce the BrdU-effect. Thymidine by itself had no effect on growth or morphology of human neuroblastoma cells. These observations are qualitatively similar to those reported with mouse neuroblastoma cells (8, 16).

Table I shows that control cells had the ChA activity, but lacked TH and COMT: however, the levels of TH and COMT became demonstrable 10 days after 5-BrdU treatment. This concentration of 5-BrdU did not change the intracellular level of cyclic AMP which was about 7 ± 2 pmole/mg protein in control cells. The addition of an equimolar of thymidine together with 5-BrdU did not reduce the BrdU-induced TH activity. Thymidine by itself did not induce the TH activity. In mouse neuroblastoma cells, 5-BrdU also increases the levels of TH, CO MT (9) and cyclic AMP (16). In addition, we have postulated (17, 18) that cyclic AMP may be involved in the regulation of TH ac-

TABLE I. Effect of 5-Bromodeoxyuridine on Tyrosine Hydroxylase (TH), Catecho-*o*-Methyltransferase (COMT) and Choline Acetyltransferase (ChA) Levels in Human Neuroblastoma Cells in Culture.[a]

Treatment	Enzyme activities (pmol/min/mg protein)		
	TH	ChA	COMT
Control cell culture	0	137 ± 16[b]	0
5-Bromodeoxyuridine (0.5 μM)	8.8 ± 1.8	74 ± 7	6.3 ± 1.9
Whole mouse caudate nucleus	20[b]	100[c]	42[d]

[a] Cells were plated in large Falcon flasks (75 cm²) and 5-Bromodeoxyuridine (0.5 μM) was added 2 days later. Enzymes were assayed 9–10 days after treatment. Fresh medium and drugs were replaced 5 and 8 days after treatment. Each value represents an average of 6–8 samples.

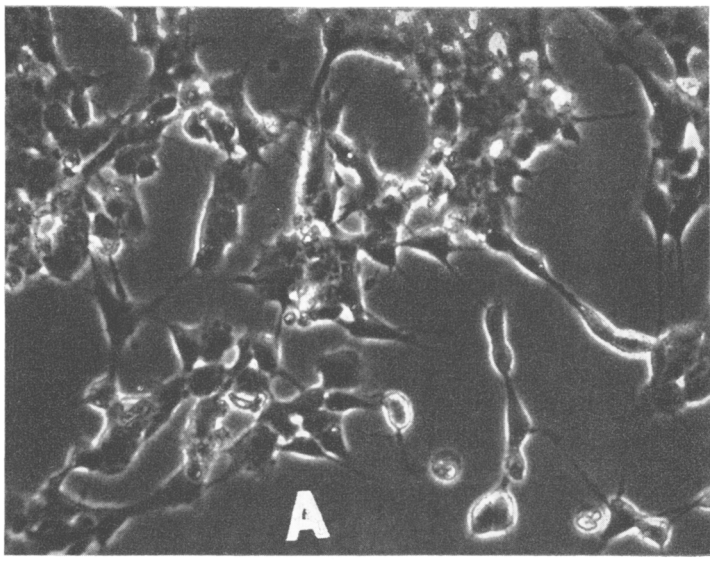
[b] SD.

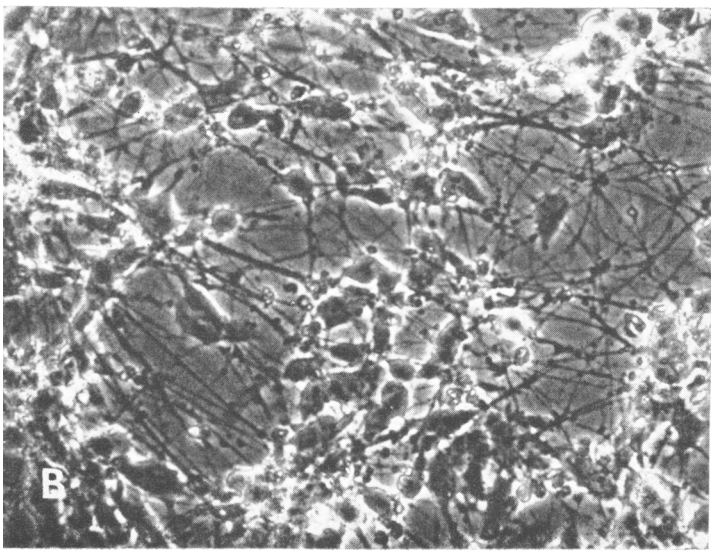
[c] Per milligram of wet caudate nucleus tissue (Waymire, J.C., personal communication).

[d] Bovine adrenals (22).

tivity in mouse neuroblastoma cells. A recent study (19) using mouse adrenal gland TH has confirmed our hypothesis. The present data suggest that cyclic AMP may not be involved in the regulation of TH level: however, our preliminary data (unpublished) indicate that dibutyryl cyclic AMP causes an elevation of TH level and morphological differentiation in human neuroblastoma cells. Hence the possible role of cyclic AMP in the regulation of TH activity and "differentiation" can not be excluded by the present study. The ChA level in human neuroblastoma cells decreased by about 50% of control after 5-BrdU-treatment. This effect is in contrast to mouse cells in which the enzyme activity increases after such treatment (9).

This study confirms our previous hypothesis (20) that both ChA and TH levels can be expressed in the same neuron. Two opposite suggestions have been made regarding the expression of these neural enzymes. Amano *et al.* have suggested that both ChA





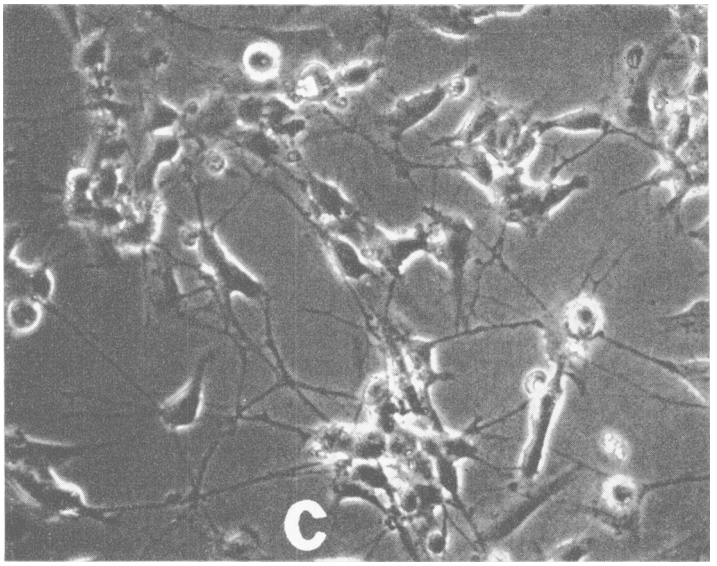


FIG. 1. Human neuroblastoma cells were plated in culture dishes (60 mm) and 5-Bromodeoxyuridine (5-BrdU, 2.5 μM) was added 3 days later. The medium and drug were changed every two days and photographs were taken 10 days after treatment. Control culture (A) shows an extensive clumping of cells, a very few of which have relatively short neurites. The 5-BrdU treated culture shows an abundance of neurites in the crowded (B) and sparsely (C) populated area of the culture. Many cells do not form neurites and continue to grow. (Mag × 131).

and TH can not express in the same neuron (21); whereas we have suggested that they can be expressed in the same neuron (20). The mechanism of action of 5-BrdU-effect on human neuroblastoma cells is unknown. It has been shown (16) that in mouse neuroblastoma cells 5-BrdU causes an elevation of the cyclic AMP level and therefore the expression of differentiated phenotype and increased TH activity after 5-BrdU treatment may be related to the cyclic AMP level. In human neuroblastoma cells, a low concentration of 5-BrdU (0.5 μM) does not change the level of cyclic AMP. Whether or not a higher concentration of this drug (1–5 μM) which is relatively more potent in causing morphological differentiation would increase the level of cyclic AMP cannot be ascertained at this time.

Mouse neuroblastoma cells attach to the flask surface more firmly after BrdU-treatment (8, 16). It has been suggested (8) that the interaction between cells and flask surface initiates the morphological differentiation. However, human neuroblastoma cells did not attach more firmly to the flask surface as evidenced by the fact that these cells are removed from the flask even without any Viokase treatment. Thus there appears to be no relationship between the interaction of cells with the flask surface and the expression of differentiated phenotype at least for this clone of human neuroblastoma cells.

Summary. 5-Bromodeoxyuridine (5-BrdU) caused morphological "differentiation" of human neuroblastoma cells in culture, as shown by the formation of long neurites. This clone had a high level of choline acetyltransferase (ChA), however, 5-BrdU treatment allowed the expression of TH and COMT without any change in the cyclic AMP level. The addition of an equimolar of thymidine together with 5-BrdU did not reduce the BrdU-effects on morphological differentiation and TH level. The ChA activity decreased in BrdU-treated cells. This study indicates that the ChA and TH activities can be expressed in the same neuron.

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