EFFECTS OF 5-(3,3-DIMETHYL-1-TRIAZENO) IMIDAZOLE-4-CARBOXAMIDE [NSC 45388, DTIC] ON NEUROBLASTOMA CELLS IN CULTURE

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SUMMARY: The effects of 5-(3,3-dimethyl-1-triazeno) imidazole-4-carboximide (DTIC) on morphological and biochemical parameters of differentiation were studied in mouse neuroblastoma cells in culture. DTIC (10 μg/ml) did not induce formation of neurites in the cells but inhibited cell division, and produced a marked increase in cell size and in activity of three enzymes (tyrosine hydroxylase, choline acetyltransferase and acetylcholinesterase) involved in neurotransmitter metabolism. These effects were apparently not related to an increase in the intracellular level of cyclic AMP.

INTRODUCTION

The antitumor activity of 5-(3,3-dimethyl-1-triazeno) imidazole-4-carboxamide (NSC 45388, DTIC) has been demonstrated against animal and human neoplasms (1-5). The mechanism of action of this drug is unknown; however, several hypotheses have been proposed (6-7) including: (a) inhibition of DNA synthesis by acting as a purine analog; (b) acting as an alkylating agent by releasing a mustard radical; and (c) interaction with SH-groups. It is believed that the malignancy of nerve cells is the result of an abnormal regulation of differentiation (8). Furthermore, active induction of differentiation in neuroblastoma cells has been linked with reduction of oncogenicity (9). Therefore, it was of interest to study the effects of DTIC on certain biochemical and morphological parameters of differentiation of neuroblastoma cells in culture. It was of particular interest to determine if DTIC inhibited cyclic nucleotide phosphodiesterase activity. DTIC contains an imidazole group like that possessed by 4(3-butoxy-

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4-methoxybenzyl)-2-imidazolidinone which has been shown to inhibit the activity of cyclic AMP phosphodiesterase in rat erythrocytes (10) and in mouse neuroblastoma cells (11).

MATERIALS AND METHODS

Culture Conditions

The procedures for culturing and maintenance of mouse neuroblastoma cells were previously described (12). The neuroblastoma clone (NBP_2) used in this study has both tyrosine hydroxylase and choline acetyltransferase (13). Cells (0.5 X 10^6) were plated in Falcon plastic flasks (75 Cm_2) and DTIC, dissolved in water, was added to the flasks 24 hours after plating. Medium was replaced and the drug added on days 2 and 3. Control cultures were treated with an equivalent volume of solvent on the same schedule. On day 4 cells were harvested using 0.25 percent Viokase solution, isolated by centrifugation (70 X g for 6 min) and washed twice in medium. An aliquot was used for determining cell number in the Coulter counter and the remaining sample was assayed for enzymatic activity and protein content.

Biochemical Enzymatic Analyses

The cells were homogenized in 0.1% Triton X-100 (Beckman) and tyrosine hydroxylase activity was measured according to the method of Waymire *et al.* (14). This procedure involves the recovery and assay of $^{14}CO_2$ after quantitative decarboxylation with hog kidney aromatic-L-amino acid decarboxylase of carboxyl labeled dopa formed from carboxyl labeled tyrosine. The 200 µl incubation mixture included 40 µmole sodium acetate (pH 6.1); 0.2 µmole ferrous sulfate; 0.4 µmole 6-7-dimethyl-5,6,7,8-tetrahydropteridin; 10 µmole mercaptoethanol; 0.02 µmole 1-^{14}C-L-tyrosine (10 Ci/mole) (New England Nuclear Corp.) and 10 units hog kidney aromatic-L-amino acid decarboxylase containing 0.005 µmole pyridoxal phosphate. After 20 min incubation at 37° the reaction was stopped by the injection of 200 µl 10% trichloracetic acid.

The $^{14}CO_2$ was collected in 0.2 ml of NCS (R) solubilizer that was contained in a plastic well (Kontes Co.) suspended from the rubber septum covering the flask opening. Wells were transferred to toluene scintillation fluid and the radioactivity was counted by liquid scintillation spectrometry.

Choline acetyltransferase was analyzed by the method of Fellman (15) as modified by Prasad et al. (13). The procedure involves conversion of choline to acetylcholine by choline acetyltransferase in the presence of ^{14}C-acetyl-coenzyme A. ^{3}H-acetylcholine was added after incubation as an internal standard to correct for recovery. ^{14}C-acetylcholine was precipitated by KI_3.

The activity of acetylcholinesterase was determined colorimetrically by the method of Ellman et al. (16) which measures the rate of hydrolysis of the substrate acetylthiocholine at 37°. The final reaction mixture consisted of 2.8 ml buffer prepared with 0.07 M Na_2 HOP_4 and 0.07M KH_2PO_4, 100 µl cell suspension (10^5), 100 µl dithiobisnitrobenzoic acid (0.01M) and 20 µl acetyl-thiocholine iodide (0.075M).

Cyclic 3',5'-AMP phosphodiesterase activity was analyzed according to the method of O'Dea et al. (17). In brief, the procedure involves conversion of ^{3}H-cyclic AMP to ^{3}H-5'-AMP which is then converted to ^{3}H-adenosine by nucleotidase. The radioactivity of ^{3}H-adenosine was measured by liquid scin-tillation spectrometry.

Cyclic 3',5'-AMP levels were measured according to Gilman's method (18). Protein was determined by the method of Lowry et al. (19).

RESULTS AND DISCUSSION

Treatment of the mouse neuroblastoma cells with DTIC (10 µg/ml) for three days did not induce formation of neurites. However, the cells became large and cell division stopped (data not shown). This effect was reflected in an elevated protein content of DTIC-treated cells (Table 1). There was no significant cell death during the period of observation as evidenced

TABLE 1. EFFECT OF 5-(3,3 DIMETHYL-1-TRIAZENO) IMIDAZOLE-4-CARBOXAMIDE (DTIC) ON THE ENZYME ACTIVITY IN NEUROBLASTOMA CELLS.

Cells were treated with DTIC (10 μg/ml) for a period of 3 days. Enzyme activity was determined according to the procedure described in the section of material and method. Each value represents an average of 6 samples.

Treatment	TH (pmole dopa formed/ hr/10^6 cells)	ChA (pmole acetylcholine formed/hr/10^6 cells)	AChE (pmole acetylthiocholine hydrolysed/hr/10^6 cells)	Protein (mg/10^6 cells)
Control	49 ± 5	530 ± 12	61,800 ± 12,600	0.30
DTIC	368 ± 32	4890 ± 77	203,400 ± 9,600	0.85

a Standard error of mean.

TH Tyrosine hydroxylase activity.

ChA Choline acetyltransferase activity.

AChE Acetylcholinesterase activity

by the fact there were only a few floater cells in the medium. The increased number of floater cells in the medium is a good relection of cytotoxicity of neuroblastoma cell in culture. The viability of attached cells as determined by the uptake of trypan blue (0.01% in saline) was similar (98%) in both control and DTIC-treated culture (3 days after treatment). The cell number in control and DTIC treated culture (50,000 cells were plated in 60 mm dish, and the drug was added 24 hrs after plating. The drug and medium were changed very day) 3 days after treatment was 19 ± 1 x 10^5 and 1.7 ± 0.22 x 10^5, respectively. The DTIC-treated cells attached to the dish surface more firmly than control cells.

DTIC-treated neuroblastoma cells had higher activities of tyrosine hydroxylase, choline acetyltransferase and acetylcholinesterase in comparison to controls (Table 1). The elevation in activity of these three enzymes exceeded the increase in protein content and the activity of the enzymes expressed per mg protein was also significantly higher in the DTIC-treated cells than controls (t-test; $p<0.05$). Several studies using neuroblastoma cells in culture have shown that the activity of these enzymes involved in neurotransmitter metabolism can be increased in the absence of morphological differentiation (8, 12, 21).

DTIC did not affect the cyclic AMP phosphodiesterase activity in homogenates of mouse neuroblastoma cells ($\bar{X}$, DTIC-treated=290 ± 41; $\bar{X}$, control= 305 ± 30; pmole/min/mg protein). DTIC also did not change the intracellular level of cyclic AMP in neuroblastoma cells in culture ($\bar{X}$,DTIC=11 ± 1.5; $\bar{X}$,control=10 ± 2.0 pmole/mg protein). Considerable data have been accumulated to indicate that the increased level of cyclic AMP is not always necessary for an increase in the activity of acetylcholinesterase or choline acetyltransferase (8,12,22). However, in mouse neuroblastoma cells an increase in tyrosine hydroxylase activity was linked with an increase in cellular cyclic AMP (8, 23). This study reports for the first time that an increase in tyrosine hydroxylase in mouse neuroblastoma can occur without any change in the cyclic AMP level. However, in a previous study (24) using human neuroblastoma cells in culture (IMR-32 line), it was observed that 5-bromodeoxyuridine increases tyrosine hydroxylase activity without any change in the level of cyclic AMP.

The present results provide evidence that the mechanisms of DTIC-effect on neuroblastoma cells does not involve any change in the cyclic AMP level. The data derived from DTIC treatment of neuroblastoma cells also support our earlier conclusions (8), namely, (a) the expression of biochemical differentiated function can be increased in the absence of neurite formation and vice versa, and (b) the expression of some differentiated functions can be

increased in the absence of any change in the intracellular level of cyclic AMP. Results of the present and previous (8, 23) studies suggest that tyrosine hydroxylase activity in neuroblastoma cells may be under at least two modes of regulation, one of which involves changes in cyclic AMP levels.

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