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Effects of Carbaryl on Differentiated and Undifferentiated Neuroblastoma Cells: Inhibition of Growth Rates and Direct Cell Toxicity

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In vitro toxicity tests, although an attractive alternative to animal tests, frequently yield discrepant values from those obtained in vivo (Ekwall 1980; Murakami and Fukami 1983; Shea and Berry 1983a, 1983b). Factors implicated in this discrepancy include cell types chosen, concentration of test compounds, external environmental stress not present in vitro, and differences in culture media components (Mummery et al. 1983; Shea 1985; Murakami and Fukami 1983). Furthermore, the majority of in vitro cytotoxicity tests, based on simple cell counts, do not readily distinguish between the relative contributions of cell death/detachment and inhibition of cell division (Tardiff 1978; Mummery et al. 1983). The relative contribution of these two parameters was demonstrated in an earlier study by time-lapse cinematography, which showed that while certain compounds affected only growth rate, others exerted equal or greater effects on cell death/detachment (Mummery et al. 1983).

In the present study, both the inherent discrepancies in comparisons of in vivo and in vitro toxicity levels, as well as the relative contributions of growth inhibition and cell death were addressed in an in vitro toxicity test of carbaryl on mouse NB2a neuroblastoma cells. These cells normally grow exponentially but can be induced to differentiate, which results in a dramatic reduction in growth rate (Shea et al. 1985). The data demonstrate that carbaryl is nearly twice as toxic to undifferentiated cells as it is to differentiated cells, but that the same relatively low concentration of carbaryl completely inhibits the growth rate of both populations of cells.

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MATERIALS AND METHODS

Mouse NB2a neuroblastoma cells, a clonal line of C1300 (Augusti-Tocco and Sato, 1969) were plated at $1x10^4$cells/well in 24-well trays in Dulbecco's Modified Eagle's medium containing 10% fetal calf serum and 25mcgs/mL Gentamycin (Gibco, New York). The cultures were maintained in a 37 C humidified atmosphere of 95% air and 5% CO_2. Twenty-four h after plating the medium was replaced with fresh medium containing 1mM N^6,O^2-dibutyryl adenosine 3',5' cyclic monophosphate (dbcAMP; Sigma Chem. , St. Louis, MO). This treatment has been shown to inhibit the growth rate and to induce bipolar axonal neurites in virtually all of the cells (Shea et al. 1985; Fischer et al. 1986). These conditions of plating permitted logarithmic growth of control cells over at least 7 days without crowding of the culture well.

Stock solutions of carbaryl (technical grade, 99.07% pure, a gift of Union Carbide) were prepared in 100% ethanol and were serially diluted into culture medium. The final concentration of ethanol in culture medium was 0.1%, which was also included in control cultures, and has been shown to have no detectable effect on cell growth and differentiation of these cells (Shea et al. 1985). Differentiated and undifferentiated control cultures received carbaryl 48 h after treatment with dbcAMP. An additional 48 h later cultures were examined for carbaryl toxicity both by phase contrast microscopy and determination of cell numbers.

Quadruplicate differentiated and control cultures were harvested following dbcAMP treatment and following carbaryl treatment and cell numbers were counted in a hemacytometer. Determinations of cell numbers varied from 5-10% among replicate cultures. Trypan blue was added to cell suspensions in order to determine cell viability. For growth rate determinations, the mean number of treated and/or differentiated cells at a given point was divided by the mean number of control cells. Values for treated cells were compared with those obtained for control cultures to ascertain the respective effects of dbcAMP and carbaryl.

RESULTS AND DISCUSSION

Mouse NB2a neuroblastoma cells were induced to differentiate by treatment with 1mMdbcAMP. Within 48 h after treatment, virtually all of the treated cells had extended long bipolar neurites (data not shown, see Shea et al. 1985). Comparison of numbers of cells in cultures of differentiated and undifferentiated cells revealed 24% fewer cells in differentiated cultures 48 h after treatment and 77% fewer cells 96 h after treatment (Fig. 1).

Calculation of the rates of cell growth under both conditions demonstrates that while the rate of growth of undifferentiated cells increases dramatically, the rate of growth of differentiated cells increases only marginally following differentiation (Fig. 2). The effect of carbaryl can therefore be examined on two populations of the same cell type: an undifferentiated population which has a rapid rate of growth, and a differentiated population in which there is a much slower rate of growth.

The toxic effects of carbaryl, as observed for both cell populations by phase-contrast microscopy, were an accumulation of phase-dense granules and detachment of cells at the highest concentrations tested. Retraction of neurites in the differentiated cultures was not observed. The toxicity of various concentrations of carbaryl 48 h after treatment, as ascertained by numbers of cells/culture, is shown in Fig. 3. The lowest concentation tested, 10^{-6}M, did not result in any inhibition in cell numbers in either undifferentiated or differentiated cultures. Carbaryl at 10^{-5}M inhibited cell numbers by 62% in undifferentiated cultures but only by 35% in differentiated cultures. Carbaryl at 10^{-4}M inhibited cell numbers by 85% in undifferentiated cultures but only by 52% in differentiated cultures. Carbaryl at $5x10^{-4}$M resulted in 100% inhibiton of undifferentiated cultures; this level of inhibiton was not observed for differentiated cultures until 10^{-3}M. Carbaryl is considerably more toxic to undifferentiated than to differentiated neuroblastoma cells, with an extrapolated value of 50% cell death/detachment observed at $8x10^{-5}$M for undifferentiated cells, while 50% cell death/detachment was not observed for differentiated cells until $1.5x10^{-5}$M.

Comparison of the relative effects of carbaryl on the growth rate of differentiated and undifferentiated cells is shown in Fig. 4. Carbaryl

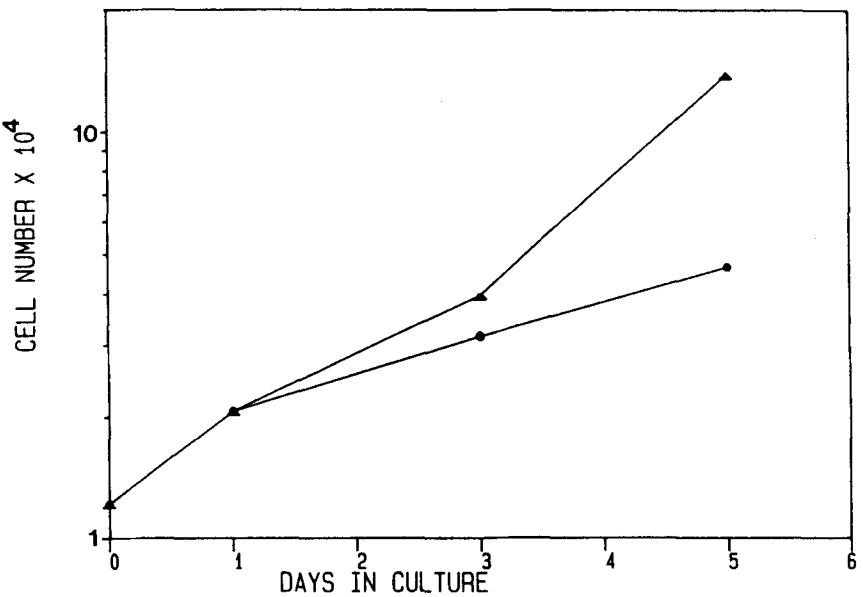

Figure 1: Effect of dbcAMP on numbers of undifferentiated and differentiated NB2a cells/culture. NB2a cells were plated in 24-well trays and 24 h later half of the cultures received 1mM dbcAMP. Quadruplicate cultures were trypsinized and counted in a hemacytometer at 24 hr intervals after plating. Triangles represent undifferentiated cells; circles represent differentiated cells.

at 10^{-6}M had no effect on the growth rate of either cell population. An effect was first observed at 10^{-5}M carbaryl. Undifferentiated cells demonstrated a growth rate of 1.32 at this concentration, while differentiated cells demonstrated a growth rate of 0.95. Since the growth rate in this instance is defined as the mean number of cells following treatment divided by the mean number of cells prior to treatment, a growth rate of approximately 1 indicates that there has been no net growth of cells. This concentration of carbaryl has therefore virtually eliminated any net growth in both cell populations. Concentrations above this resulted in negative growth rate values, which are reflections of direct toxicity leading to death/detachment, abrogating specific examinations of growth rates.

It is difficult to completely separate the parameters of toxicity and growth rate inhibition in assessing the effects of a cytotoxic

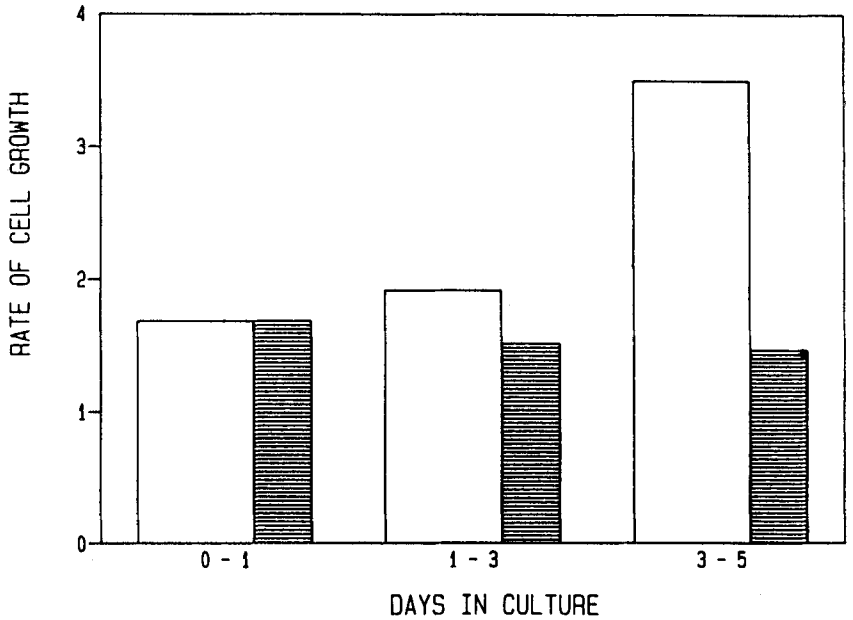

Figure 2: Effect of dbcAMP on growth rate of differentiated and undifferentiated NB2a cells. Differentiated and undifferentiated NB2a cultures were trypsinized and counted in a hemacytometer at days 0, 1, 3 and 5 after plating, with dbcAMP treatment on day 1 after plating. The growth rate was determined as described in Materials and Methods. Clear bars represent undifferentiated cultures; shaded bars represent differentiated cultures.

compound. However, the experimental design utilized herein, namely the establishment of two populations of the same cell type, one of which has a reduced growth rate as a result of differentiation, permits a degree of independent evaluation of these parameters. Carbaryl was more toxic to undifferentiated NB2a cells at all concentrations tested as shown by direct comparison of cell numbers. However, examination of the rates of growth of cells in the presence of carbaryl revealed that 10^{-5}M resulted in a virtual cessation of growth for both differentiated and undifferentiated cells.

A previous study (Mummery et al. 1983) utilized time-lapse cinematography of growing cell populations to address whether indeed growth inhibiton in neuroblastoma cultures treated with cytotoxic compounds could be attributed to cell death or impaired progression through the cell cycle. It was observed that 4/8 compunds exerted nearly all of their inhibition at the level of retardation of cell cycle progression, while another 4 exerted equal

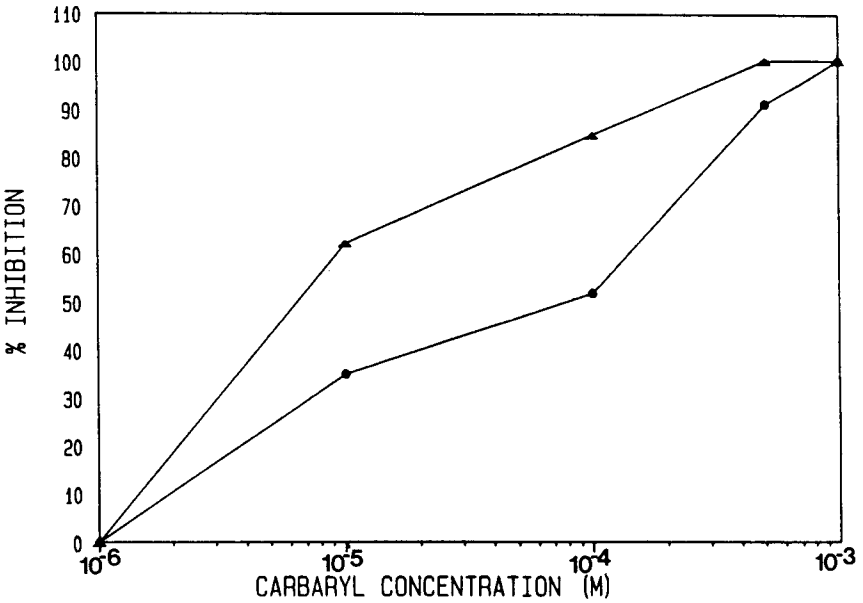

Figure 3: Effect of carbaryl on numbers of differentiated and undifferentiated NB2a cells/culture. Differentiated and undifferentiated NB2a cultures received various concentrations of carbaryl. Forty-eight h later cultures were trypsinized and counted in a hemacytometer. Triangles represent undifferentiated cells; circles represent differentiated cells.

or greater effects on cell death/detachment. The results of the present study would place carbaryl in the latter group, since both decreased cell division as well as increased cell death/detachment were observed.

The increased sensitivity of undifferentiated NB2a cells to carbaryl is likely to be a reflection of mitotic events, being more stressful to the cells' homeostasis; i.e., a differentiated cell may be programmed to operate on somewhat more maintenance-oriented pathways and is therefore capable of tolerating the additional stress of a xenobiotic compound. This phenomenon may prove important in comparisons of in vivo and in vitro toxicity testing. In attempts to construct cell culture models as alternatives to animal testing (Walton and Buckley 1978; Exwall 1980; Tardiff 1978; Nardone 1977; Mummery et al. 1983), utilization of primary cell cultures, or manipulation of established cell lines to induce a differentiated state, may assist in

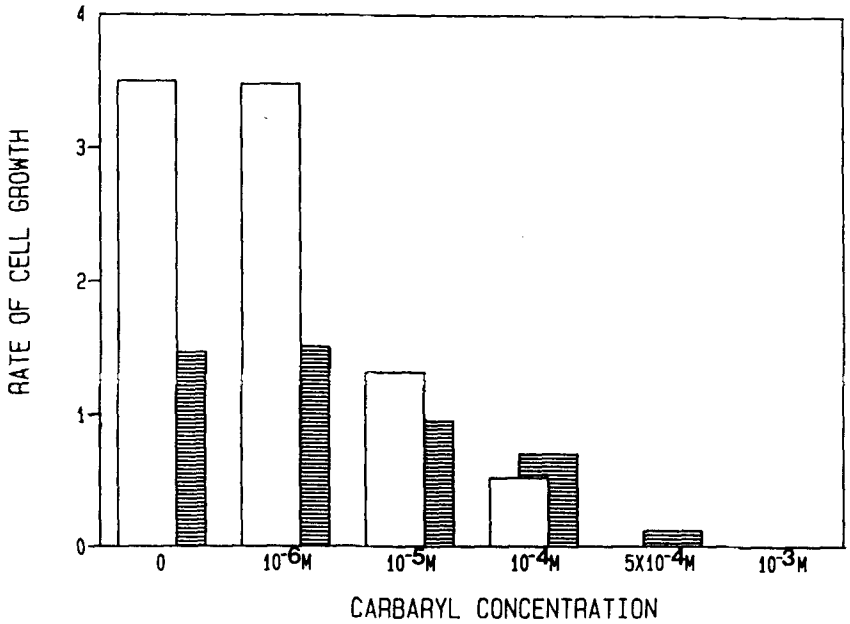

Figure 4: Effect of carbaryl on growth rate of differentiated and undifferentiated NB2a cells. Differentiated and undifferentiated NB2a cultures were trypsinized and counted in a hemacytometer 48h after treatment with carbaryl. The growth rates were determined as described in Materials and Methods. Clear bars represent undifferentiated cultures; shaded bars represent differentiated cultures.

the evaluation of in vitro data for assessment of the environmental impact of a compound. The increased toxicity of carbaryl to rapidly dividing, undifferentiated cells as compared to differentiated cells also has implications of the potential dangers of this compound to embryonic or developing organisms.

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