BRIEF NOTES

ENUCLEATED NEUROBLASTOMA CELLS FORM NEURITES WHEN TREATED WITH DIBUTYRYL CYCLIC AMP

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INTRODUCTION

Cultured cells cloned from the C1300 neuroblastoma have been shown by several laboratories to extend neurites (1–6) and to accumulate nerve-specific enzymatic activities (7) when grown under conditions in which cell division is inhibited. Study of this system has been stimulated by the hope that it might serve as an in vitro model of nerve cell differentiation. Many of the neurite-inducing agents, including serum deprivation (3), prostaglandins (4), theophylline (5), and analogues of cAMP[1] including dibutyryl cAMP (B_2cAMP), are thought to act principally by raising the intracellular concentrations of cAMP. Division is inhibited in all these cases, as well as in non-neural cell cultures treated with cAMP analogues (8).

The observed stimulation of neurite extension in cAMP-treated neuroblastoma cultures might be explained in either of two ways. The "direct" explanation is that cAMP itself stimulates neurite outgrowth, and inhibits division only coincidentally. The "indirect" hypotheses maintains that since neurites grow during interphase and are retracted during mitosis (3), cAMP *permits* neurite extension by stopping the cell cycle in interphase. The action of cAMP under either hypothesis might or might not require interaction with or alteration of nuclear activity.

We report here that enucleated neuroblastoma cells extend neurites when treated with B_2cAMP. Our results demonstrate that at least the early stages of neurite extension do not require alteration of intranuclear events, and add support to the "direct" explanation of cAMP-induced neuroblastoma differentiation.

[1] *Abbreviations used in this paper:* B_2cAMP, dibutryl cAMP; cAMP, adenosine 3'-5'cyclic monophosphoric acid; CB, cytochalasin B; cGMP, guanosine 3'-5'cyclic monophosphoric acid; CHO, Chinese hamster ovary cells.

MATERIALS AND METHODS

Materials

Serum and tissue culture media were obtained from Grand Island Biological Co., Grand Island, N. Y., plasticware from Falcon Plastics, Div. of B.-D. Laboratories, Los Angeles, Calif., B_2cAMP, cGMP and butyric acid from Sigma Chemical Corp., St. Louis, Mo., and cytochalasin B from Aldrich Chemical Co., Inc., Milwaukee, Wis.

Cell Line

Clone Neuro-2*a*, derived from the spontaneous mouse C1300 neuroblastoma by Klebe and Ruddle (9), was grown in commercially treated plastic flasks in Dulbecco-Vogt modified Eagle's medium containing 8–10% gamma globulin-free newborn calf serum and antibiotics under 10% CO_2 at 37°C. Cultures were passaged weekly.

Enucleation

Cells were enucleated by a modification of the technique of Prescott et al. (10). Plastic disks were cut from the bottoms of tissue culture dishes, sterilized by UV irradiation, and placed into 2 ml of growth medium. 1–3 $\times 10^5$ cells were then added and allowed to attach overnight. Cytochalasin B (CB) was then added to a final concentration of 10 μg/ml from a stock solution of 1 mg/ml in dimethyl sulfoxide. After 1 h of incubation at 37°C, the disks were placed into sterile centrifuge tubes containing 8 ml of growth medium and 2.5 μg/ml of CB,

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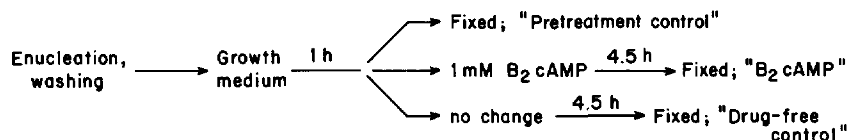

FIGURE 1 Protocol for production of pretreatment control, drug-free control, and B_2cAMP-treated cultures as described in text.

and were centrifuged for 10 min at 7000–9000 *g* in an RC-2B centrifuge using an SS-34 rotor prewarmed to 37°C. This lower concentration of CB was used for the centrifugation step because the cells detached from the plastic disk if spun at a higher concentration of the drug. Centrifugation of the CB-treated cells caused the nuclei to separate from the cytoplasmic residues, which remained attached to the plastic. Giemsa-stained preparations usually contained 85–95% enucleated cells; disks less satisfactory than this were not used for analysis.

After the centrifugation, the disks were washed twice in drug-free Hanks salt solution, placed into 2 ml of fresh growth medium, and incubated for 1 h to permit recovery from the effects of the CB. At this point (see Fig. 1), one group of disks was fixed in 3:1 ethanol-acetic acid, stained in Fisher's Giemsa, washed three times in Gurr's buffer (pH 6.8), and air dried. These cultures served as a pretreatment control. A second group was treated, after 1 h in drug-free medium, with the agent under investigation (usually 1 mm B_2cAMP as described below) for a period of 4.5 h before fixation and staining. A third group, the drug-free control, was incubated for the additional 4.5 h in growth medium alone. The incubation period we used, 4.5 h, was sufficient to permit the drug-induced morphological alterations. Longer incubation times were not so instructive, because of the morphological degeneration which occurred even in control cytoplasms upon longer (e.g., 12-h) incubation.

Scoring of Morphology

After staining, random microscope fields were photographed and then evaluated by scorers who were blind to the treatment conditions. Randomly chosen cells (no more than 5 per field and 40 per disk) were scored for number of neurites, with a neurite defined arbitrarily as an extension from the cell body longer than the average diameter of that soma. Cells which had at least one neurite were also scored for neurite length, branching, and extent of neurite varicosity formation.

RESULTS

The clone of neuroblastoma cells which was used in our experiments, Neuro-2*a*, is highly pleomorphic in logarithmic growth. A given culture always contains, in close proximity, dividing round cells, flat cells without processes, and cells which possess neurite-like extensions which vary considerably in length and complexity. Similarly, cells which have been enucleated and allowed to recover for 1 h in growth medium (pretreatment controls, as described above) showed a wide spectrum of morphologic types. A representative field is presented in Fig. 2 *a*. Cells which were incubated for an additional 4.5 h in the absence of added drug (drug-free controls) appeared similar to the pretreatment cells (Fig. 2 *b*). Addition of 1 mM B_2cAMP at the beginning of this 4.5-h period, however, seemed to increase the number, length, and complexity of the neuronal processes in the culture (Fig. 2 *c*).

Since this morphological change produced by B_2cAMP did not occur in every enucleated Neuro-2*a* cell in a given population, we undertook statistical analysis to test our initial impressions. Table I demonstrates that the fraction of cells with at least one neurite is significantly greater in the B_2cAMP-treated cells than in either of the two controls.

B_2cAMP-treated cells also differed from both control populations in that their neurites, when present, were longer in proportion to the cell body diameter, and contained, on average, a greater number of bead-like varicosities. These differences were statistically significant (χ^2 test) at better than the 0.025 level.

We have conducted preliminary experiments to see if the effect of B_2cAMP might have been mediated by contamination of the commercial preparation with butyrate, as has been suggested in at least one similar study (11). In three separate experiments, 1 mM sodium butyrate in place of the B_2cAMP produced cytoplasm morphologically indistinguishable from that of either control population.

Because cGMP has been reported to antagonize the effects of cAMP analogues in several systems, including one system (12) arguably involving microtubule polymerization, we tested the ability of cGMP to oppose the morphological changes induced by B_2cAMP in neuroblastoma cytoplasms. In three experiments in which cGMP was added to

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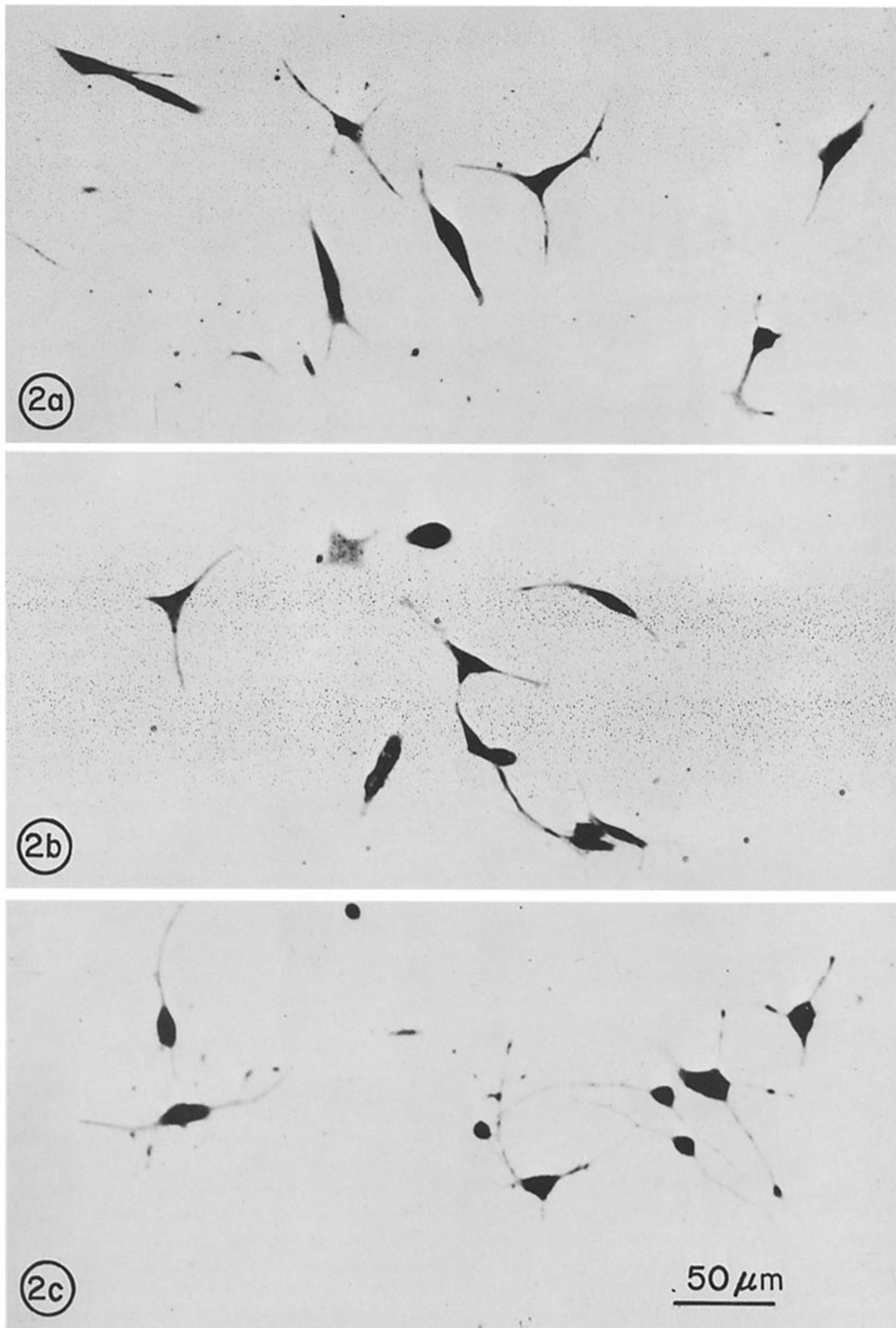

FIGURE 2 Selected, representative fields of fixed, Giemsa-stained Neuro-2*a* cytoplasmic residues prepared as described in text and in Fig. 1. In (*a*), pretreatment control; (*b*), drug-free control; (*c*), B_2cAMP-treated. The bar indicates 50 nm. × 310.

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Table I

Frequency of Occurrence of Neuronal Processes in Treated and Untreated Cells

Cell type	Fraction with neurites		
	n	No neurites	One or more
Pretreatment	125	49 (39)	76 (61)
Drug-free	120	45 (38)	75 (62)
B_2cAMP	150	28 (19)	122 (81)

Comparison	χ^2 analysis	
	χ^2	Probability
Pretreatment vs. drug-free	0.06	$0.90 > P > 0.80$
Pretreatment vs. B_2cAMP	14.26	$0.00025 > P$
B_2cAMP vs. drug-free	12.07	$0.0005 > P > 0.00025$

Numbers in parentheses denote percentages.

B_2cAMP-treated cultures in up to fourfold molar excess, neurite extension occurred which was superficially indistinguishable from that of cultures treated with B_2cAMP alone.

DISCUSSION

We have shown that the administration of 1 mM B_2cAMP to enucleated neuroblastoma cells 1 h after enucleation causes an increase in the fraction of the cells which have neurites, compared to drug-free enucleated controls, when both populations are examined 4.5 h after B_2cAMP administration. B_2cAMP is not acting by preventing any possible enucleation-induced morphological change, since drug-treated cultures differed from pretreatment as well as from drug-free preparations.

Our results demonstrate that at least the initial phase of cAMP-induced morphological differentiation in neuroblastoma cells can occur independently of communication in either direction between the nucleus and cytoplasm. Many laboratories have demonstrated that cAMP can affect cytoplasmic events by altering the state of phosphorylation of specific proteins (reviewed in reference 13). The results of Langan (14) describing cAMP-mediated phosphorylation of histones render plausible the notion that in some systems cAMP may exert an effect on nuclear synthetic activity. Neuroblastoma transcription could conceivably be altered by cAMP, but our data rule out the hypothesis that any such changes are necessary in the early stages of morphological differentiation in these cells.

Since the work described above was completed, Schroder and Hsie (15) have reported a B_2cAMP-induced morphological alteration in enucleated Chinese hamster ovary cells, analogous to the drug's effect on intact CHO cells. It is possible that a common cytoplasmic event is occurring in both these cell types, the particular effect of which is channeled into neurite extension in neuroblastoma and elongation in CHO cells.

The indirect, permissive explanation for the stimulation by cAMP analogues of neurite outgrowth has been weakened by the findings that serum starvation can produce neurite outgrowth within 60 min in 75% of some clonal neuroblastoma populations (3), and that cAMP and B_2cAMP can produce similar morphological alterations in explanted chick spinal root ganglia despite the virtual absence of mitosis in this preparation (16). These last studies have, however, proved difficult to confirm (P. Greengard, personal communication). Our results add further support to the direct hypothesis. Enucleation effectively prevents the transition of cells from interphase to mitosis. Our results show that regardless of the effect this interruption may have on neurite extension, B_2cAMP produces additional outgrowth that cannot now be attributable to the drug's putative effect on a cell cycle already blocked.

Further investigations of the biochemical correlates and genetic controls of cAMP-mediated neurite extension in cultured neuroblastoma cells are now in progress.

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