Neuroblastoma Clones: Prostaglandin Versus Dibutyryl Cyclic AMP, 8-Benzylthio-cyclic AMP, Phosphodiesterase Inhibitors and X-Rays[1] (36408)

KEDAR N. PRASAD

Department of Radiology, University of Colorado Medical Center, Denver, Colorado 80220

Dibutyryl cyclic AMP (1), prostaglandins (2), inhibitors of phosphodiesterase (3) or X-rays (4) cause morphological differentiation of neuroblastoma cells in culture as shown by the formation of axon-like processes. These differentiated cells exhibit morphological maturation as revealed by an increase in the size of soma and nucleus. This paper shows that in the clone NBDB$^-$ prostaglandin E1 is much more effective in causing morphological differentiation than dibutyryl cyclic AMP, 8-benzylthiocyclic AMP, phosphodiesterase inhibitors or X-irradiation. In the clone NBA2(1) PGE1 is as effective as other differentiating agents.

Materials and Methods. The procedures for culturing mouse neuroblastoma cells and some morphological features have been previously described (5, 6). Neuroblastoma cells were grown as a monolayer in Falcon plastic flasks or dishes containing F12 medium with 10% agamma-globulin newborn calf serum, and penicillin (100 μg/ml) and streptomycin (100 U/ml), and were maintained at 36° in a humidified atmosphere of 5% CO_2 in air. The average doubling time of uncloned neuroblastoma cells is about 24 hr. This cell line has acetylcholinesterase but no butyrylcholinesterase activity (6). Tyrosine hydroxylase, a rate-limiting enzyme in the biosynthesis of catecholamines, is present in the primary tumor (7, 8), but is barely detectable in the long-term culture; however, this enzyme activity is restored to the initial level in the dibutyryl cyclic AMP-treated cells (9).

Neuroblastoma clone NBA2(1) was isolated from the uncloned line by a standard procedure. Dibutyryl cyclic AMP-insensitive clone (NBDB$^-$) was isolated as follows: uncloned neuroblastoma cells (200 cells) were plated in Falcon plastic dishes and dibutyryl cyclic AMP (1.0 m*M*) was added after the visible clones were formed. After 24 hr of incubation in the presence of dibutyryl cyclic AMP, clones which did not show morphological differentiation were isolated by a usual technique. The above procedure was repeated once. The clonal line (NBDB$^-$) was insensitive to dibutyryl cyclic AMP and had a much lower frequency (2%) of spontaneous differentiation. Both neuroblastoma clones, NBA2(1) and NBDB$^-$ had biochemical features similar to those of the uncloned line.

Dibutyryl cyclic AMP was dissolved in F12 medium without serum. Prostaglandin El and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO 20-1724) were dissolved in 95% alcohol. Papaverine was dissolved in 50% alcohol. Both RO 20-1724 (10) and papaverine (11) are powerful inhibitors of cyclic nucleotide phosphodiesterase. 8-Benzylthio-cyclic AMP was dissolved in 0.1 *N* ammonium hydroxide and the pH was adjusted to 8.6 by the addition of 1 *N* HCl. All drug solutions were stored in the freezer until use. Each drug was added to the neuroblastoma culture separately 24 hr after plating. Control cells were treated with an equal volume of solvent; the final concentration of alcohol in the medium

[1] This work was supported by U.S. Public Health Service Research Grant NS 09230 and DRG 1682 from Damon Runyon Memorial Fund for Cancer Research. I thank Drs. J. E. Pike of the Upjohn Company, H. Sheppard of Hoffman-LaRoche Inc. and Mr. K. Robins of ICN Nucleic Acid Research Institute for their generous supply of prostaglandin, RO 20-1724 and thiobenzyl-cyclic AMP, respectively. I thank April Montgomery and Marian Gaschler for their technical help.

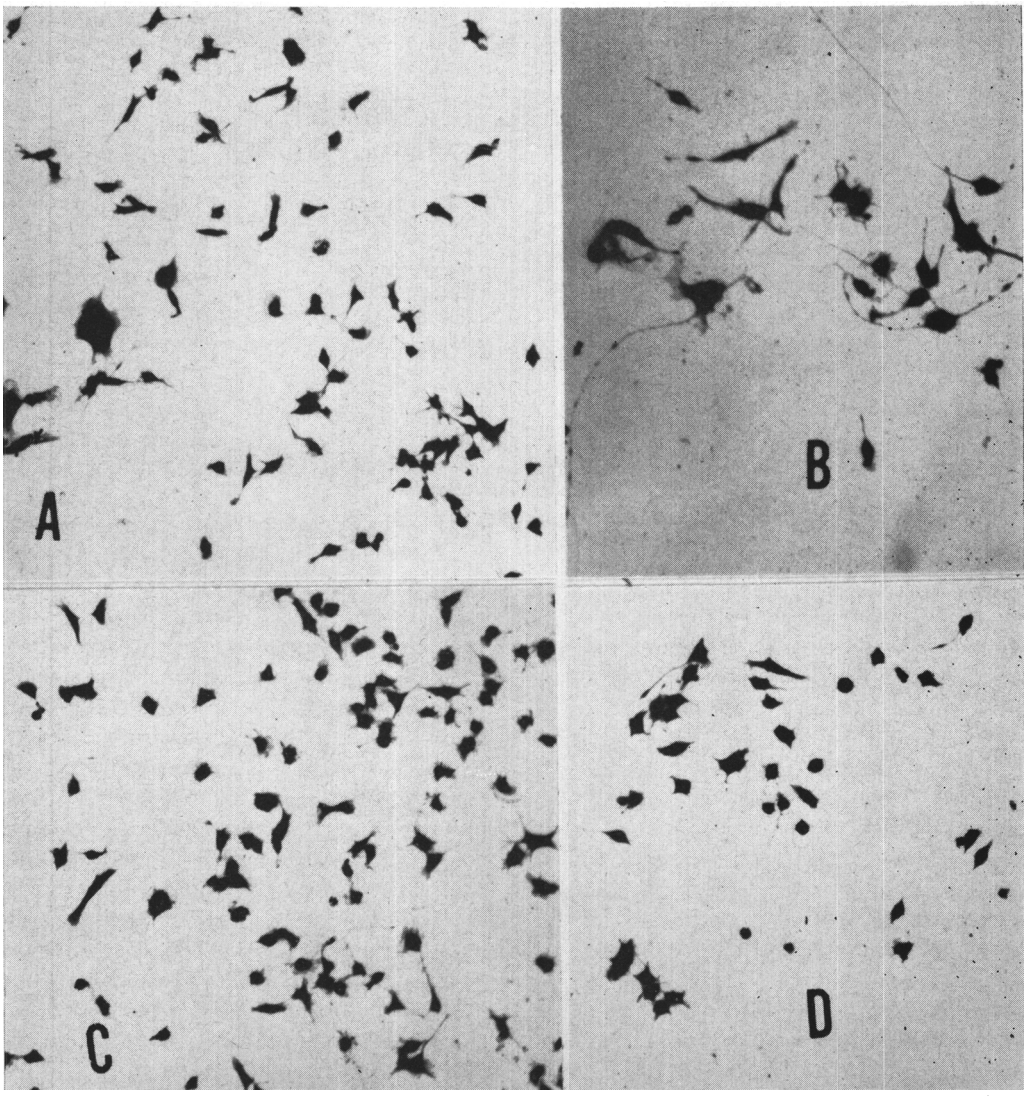

FIG. 1. Photomicrographs of neuroblastoma cells in culture. The inhibitor of phosphodiesterase (RO 20-1724) at a concentration of 200 μg/ml was added to neuroblastoma cells 24 hr after plating. Cells, while attached to the dish, were fixed in formaldehyde (1:10 dilution of concentrated formaldehyde) for 30 sec, washed twice with distilled water, stained with 0.1% cresyl violet for 2 min, rinsed quickly and air dried. Immersion oil was added to the dish before taking the photomicrograph. Control cells of NBA2(1) clone (A) are small, round and mostly undifferentiated (3 days after plating). The phosphodiesterase inhibitor-treated clone NBA2(1) shows a marked increase in the number of differentiated cells (B), whereas clone $NBDB^-$ revealed very little effect on the differentiation (D) after the same treatment. Control cells of $NBDB^-$ clone (C) have morphological features similar to those of NBA2(1) (×80).

never exceeded more than 0.01%. Radiation factors were 240 kVp, 30 mA, with added filter of 1 mm Al, 0.5 mm Cu (hvt = 1.36 mm Cu). Distance between the target and turntable was 60 cm. Dose rate was 89 rad/min. Cells were X-irrdiated (600 rads) at room temperature. The formation of axons (cytoplasmic extensions greater than 50 μm in length) is indicative of morphological differentiation, whereas an increase in the size of

TABLE I. Effect of Various Differentiating Agents on Mouse Neuroblastoma Clones.[a]

Treatment	Differentiated cells (% of total cells)	
	Clone NBA2(1)	Clone NBDB$^-$
Control	10.0 ± 2.5[b]	2.0 ± 0.5
Prostaglandin E1 (10 μg/ml)	71.0 ± 6.0	45.0 ± 3.7
Dibutyryl-cyclic AMP (1.0 m*M*)	42.0 ± 3.0	9.0 ± 2.0
Benzylthio 3′ :5′ cyclic AMP (400 μg/ml)	66.0 ± 6.6	10.0 ± 4.0
Papaverine (25 μg/ml)	69 ± 2.5	10.0 ± 4.2
RO 20-1724 (200 μg/ml)	71.0 ± 5.4	5.0 ± 2.0
X-irradiation (600 rads)	43.0 ± 3.0	9.0 ± 3.5

[a] Neuroblastoma cells (5×10^4) were plated in the Falcon plastic culture dishes (60 mm). Each drug was added separately to the culture dish 24 hr after plating and the morphologically differentiated cells were scored 3 days later. A total of 300 cells were counted and the number of morphologically differentiated cells were expressed as percent of total cells. Each value represents an average of 8–10 samples.

[b] Standard deviation.

soma and nucleus is regarded as an expression of morphological maturation (1).

Results and Discussion. The neuroblastoma clone NBA2(1) showed a marked increase in the number of morphologically differentiated (Fig. 1B) cells 3 days after the addition of phosphodiesterase inhibitor (RO 20-1724), whereas the clone NBDB$^-$ revealed relatively little effect on the differentiation (Fig. 1C) after the same treatment. The differentiated cells showed morphological maturation as shown by an increase in the size of soma and nucleus.

Table I shows that prostaglandin E1 was very effective in causing morphological differentiation in the clone NBDB$^-$ whereas dibutyryl cyclic AMP, 8-benzylthio-cyclic AMP, phosphodiesterase inhibitors or X-irradiation had very little effect on the differentiation. In the clone NBA2(1) prostaglandin E1 was as effective as other differentiating agents. The fact that the clone NBDB$^-$ is insensitive to phosphodiesterase inhibitors (papaverine and RO 20-1724) as well as to dibutyryl cyclic AMP, 8-benzylthio-cyclic AMP and X-ray on the criterion of morphological differentiation indicates that all of the above agents may have the same site of action, namely, the phosphodiesterase. From the above data the following conclusions can be made: (a) In NBA2(1) clone the initial site of action of prostaglandin (PG) E1 may be different from that of dibutyryl cyclic AMP, 8-benzylthio-cyclic AMP, inhibitors of phosphodiesterase and X-rays. This is consistent with the well known observation that PGE1 increases cyclic AMP level by stimulating adenylate cyclase. The remainder of the differentiating agents may increase the level of cyclic AMP by inhibiting the phosphodiesterase. The fact that NBDB$^-$ clone is insensitive to all differentiating agents except PGE1 indicates that the phosphodiesterase level in this clone may be too low to be of any significance in increasing the cyclic AMP level by inhibitors of phosphodiesterase. The other possibility is that the phosphodiesterase activity is not affected by inhibitors of phosphodiesterase, dibutyryl cyclic AMP, 8-benzylthio-cyclic AMP and X-irradiation. On the other hand, the adenylate cyclase of NBDB$^-$ as well as of NBA2(1) is sensitive to PGE1. (b) In the clone NBDB$^-$ the inherent biological property of a cell may be more important in initiating the morphological differentiation than the interaction between the cells and the culture dish surface. It has been reported that in some neuroblastoma clones the initiation of morphological differentiation requires a strong interaction between the cell and the surface of the culture dish (12); this does not appear to be the case in the NBDB$^-$ clone.

Summary. The effects of prostaglandin (PG)E1, dibutyryl cyclic AMP, 8-benzylthio-cyclic AMP, inhibitors of phosphodiesterase [papaverine and 4-(3-butoxy-

4-methoxybenzyl)2-imidazolidinone], and X-irradiation on two neuroblastoma clones, NBA2(1) and NBDB$^-$ were studied. In the NBDB$^-$ clone PGE1 was much more effective in causing morphological differentiation than any other differentiating agents. In the NBA2(1) clone PGE1 was as effective as the other agents. Adenylate cyclase of both clones may be sensitive to PGE1. The phosphodiesterase level in clone NBA2(1) is probably inhibited by other differentiating agents but this enzyme level in clone NBDB$^-$ either is too low to be of any significance or is insensitive to phosphodiesterase inhibitors. In clone NBDB$^-$ the biological property of the cell may be more important in initiating the morphological differentiation than the interaction between the cell and the surface of the culture dish.

1. Prasad, K. N., and Hsie, A. W., Nature New Biol. **233**, 141 (1971).

2. Prasad, K. N., Nature New Biol. **236**, 49 (1972).

3. Prasad, K. N., and Sheppard, J. R., Exp. Cell. Res. (in press).

4. Prasad, K. N., Nature **234**, 471 (1971).

5. Prasad, K. N., Cancer Res. **31**, 1457 (1971).

6. Prasad, K. N., and Vernadakis, A., Exp. Cell. Res. **70**, 27 (1972).

7. Prasad, K. N., Zambernard, J., Lasher, R., and VanWoert, M. H., Nature (London), **228**, 997 (1970).

8. Augusti-Tocco, G., and Sato, G., Proc. Nat. Acad. Sci. U.S.A. **64**, 311 (1969).

9. Prasad, K. N., Waymire, J. C., and Weiner, N., Exp. Cell. Res. (in press).

10. Sheppard, H., and Wiggan, G., Mol. Pharmacol. **7**, 111 (1971).

11. Goldberg, N. D., Lust, W. D., O'Dea, R. F., Wei, S., and O'Toole, A. G., *in* "Role of Cyclic AMP in Cell Function" (P. Greengard and E. Costa, eds.). Raven, New York; Advan. Biochem. Pharmacol. **3**, 67 (1970).

12. Schubert, D., Humphreys, S., De Vitry, F., and Jacob, F., Develop. Biol. **23**, 514 (1971).

Received Dec. 13, 1971. P.S.E.B.M., 1972, Vol. 140.