Morphologically "Differentiated" Mouse Neuroblastoma Cells Induced by Noncyclic AMP Agents: Levels of Cyclic AMP, Nucleic Acid and Protein (37493)

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Some noncyclic AMP agents such as X ray (1), serum-free medium (2) 5-bromodeoxyuridine (3), and 6-thioguanine (4) cause morphological "differentiation" of mouse neuroblastoma cells in culture similar to that produced by cyclic AMP (5–7). Consequently, the question arose whether the noncyclic AMP agents would cause morphological differentiation by increasing the intracellular level of cyclic AMP or whether they would initiate molecular changes similar to those produced by cyclic AMP. Hence, the cyclic AMP level was measured after treatment with X rays, 5-bromodeoxyuridine (5-BrdU), 6-thioguanine (6-TG), and serum-free medium (SFM). Most of the cyclic AMP-induced "differentiated" cells accumulate in the G_1 phase of the cell cycle and show a marked increase in total RNA and protein contents (8). Therefore, the question arose whether the morphologically "differentiated" cells induced by noncyclic AMP agents would show similar biochemical changes.

Materials and Methods. Two neuroblastoma clones were used in this study. The clone, $NBA_2(1)$ contains a low level of tyrosine hydroxylase (TH), but lacks choline acetyltransferase (ChA), whereas the clone $NBE^-{}_{(R)}$ contains a high level of ChA, but lacks TH. The acetylcholinesterase activity was present in both clones (9). $NBE^-{}_{(R)}$ shows a higher degree of morphological "differentiation" (65% of total) after X irradiation, when compared to $NBA_2(1)$ (40% of total). The dose requirement to produce a maximal differentiation in these clones differ by a factor of 2 (unpublished observation). Both clones are equally responsive to 5-BrdU, 6-TG, and SFM in causing morphological "differentiation." The doubling time of both clones is about 18 hr. Cells of both clones produce tumors when injected sc into male A/J mice. The procedures of culturing and maintaining neuroblastoma cells were previously described (10). Radiation factors were also earlier described (1). Cells (0.5×10^6) of clone $NBA_2(1)$ were plated in large Falcon plastic flasks (75 cm^2) and 5-BrdU (5.0 μM), 6-TG (0.5 μM), 6-mercaptopurine (0.5 μM), and 2-aminopurine (5 μM) were added separately 24 hr later. Controls were treated similarly, except no drug was added. Cells were X irradiated at room temperature 24 hr after plating. Cells of $NBA_2(1)$ and $NBE^-{}_{(R)}$ clones were given 600 and 1200 rads, respectively. These doses represent an optimal dose for inducing morphological differentiation in each clone. Prostaglandin E_1 (11) and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO20-1724), an inhibitor of cyclic AMP phosphodiesterase (12), are known to increase cyclic AMP level: therefore, PGE_1 and RO20-1724 were used for the purpose of comparison with noncyclic AMP agents. The cyclic AMP was assayed 3 days after treatment. Cells were washed twice with PDS buffer and 2 ml of cold 5% trichloroacetic acid was added. The contents were removed by a rubber policeman and homogenized. An aliquot was taken for protein determination by the method of Lowry *et al.* (13), and the remainder was used to determine cyclic AMP according to Gilman's method (14).

To determine the total nucleic acid and protein contents, neuroblastoma clone $NBE^-{}_{(R)}$ was used. Cells were plated in large Falcon plastic flasks (75 cm^2) and treated with SFM, 5-BrdU (5 μM), 6-thioguanine (0.5 μM), and X ray (1200 rads, single dose) 24 hr later. The medium and

TABLE I. Effect of Various Agents on the Cyclic AMP Level of Mouse Neuroblastoma Cells in Culture.[a]

Treatment	Cyclic AMP level (pmol/mg protein)
Control	12 ± 1.5[b]
5-bromodeoxyuridine (5.0 μM)	21.8 ± 1.7
Serum-free medium	22.5 ± 2.2
X irradiation (600 rads)	13.5 ± 1.8
6-thioguanine (0.5 μM)	14.4 ± 2.1
6-mercaptopurine (0.5 μM)	13.9 ± 2.1
2-aminopurine (5.0 μM)	10.3 ± 1.4
Butyric acid (0.5 mM)	22.0 ± 3.2
Prostaglandin E_1 (10 μg/ml)	47.1 ± 5.3
4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (200 μg/ml)	42.3 ± 4.4

[a] Cells (0.5×10^6) were plated in large Falcon plastic flasks and the treatment was started 24 hr later. Fresh growth medium and drug were changed 2 days after treatment, and the cyclic AMP was assayed according to Gilman's method (14). Each value represents an average of 6–9 samples.

[b] SD.

drug were changed 2 days after treatment because the neuroblastoma cells produce lactic acid at a relatively high rate (15). A cell suspension was prepared 3 days after treatment using 0.25% Viokase solution. An aliquot was used for determining cell number in the Coulter counter, and the remaining sample was used for nucleic acid and protein assay. Nucleic acid was extracted by the method of Schneider (16). The DNA and RNA contents were determined by the method of Burton (17) and by the orcinol method of Cerrioti (18), respectively. The data were expressed as pg DNA/cell, pg RNA/cell, and pg protein/cell.

Results and Discussion. Table I shows that 5-BrdU and SFM increased cyclic AMP level by about twofold in a clone NBA_2(1), whereas, 6-thioguanine and X irradiation did not. X irradiation (1200 rads) which produces a maximal differentiation (65% of total cells) in clone $NBE^{-}{}_{(R)}$ also did not change the cyclic AMP level. As expected, PGE_1 and R020-1724 increased cyclic AMP level by fourfold. The above data suggest the following: (a) 5-BrdU and SFM induce morphological "differentiation" probably via cyclic AMP; and (b) X ray and 6-thioguanine may bypass the step involving the elevation of cyclic AMP for the expression of differentiated phenotype. Indeed, it has been suggested (1, 4–7) that X ray, 6-thioguanine, and cyclic AMP promote the organization of microtubules and microfilaments, because vinblastine sulfate and cytochalasin B, which are known to inhibit the assembly of microtubules and microfilaments, respectively, completely block the expression of differentiated phenotype induced by above agents. Since X ray and 6-thioguanine are not naturally occurring substances, the present data did not contradict our working hypothesis that cyclic AMP may be involved in the "differentiation" of mouse neuroblastoma cells. It is interesting to note that butyric acid, a degradative product of dibutyryl cyclic AMP solution, which reversibly blocks cells in the G_1 phase of the cell cycle (8) without the expression of morphological "differentiation," also increased cyclic AMP by about two fold (Table I). This indicates that an elevation of cyclic AMP and inhibition of cell division are not sufficient for the expression of the differentiated phenotype. It is possible that butyric acid interferes with the subsequent steps involved in the expression of differentiated phenotype. Indeed, we have recently shown (19) that prostaglandin-induced elevated level of cyclic AMP does not allow the expression of differentiated phenotype if the subsequent steps involving the assembly of microtubules and microfilaments are inhibited by vinblastine sulfate and cytochalasin B, respectively.

Table II shows that the DNA content of SFM- and 6-thioguanine-treated cells did not significantly change, indicating that the relative distribution of "differentiated" cells throughout the cell cycle may be similar to that of controls. It has been generally presumed that cells accumulate in the G_1 phase of the cell cycle when SFM is added. This does not appear to be the case in this neuroblastoma clone. Cells treated with 5-BrdU only had about one-third the DNA of the control cells. This is interpreted to mean that most of the 5-BrdU-treated cells are accumulated in the G_1 phase of the cell cycle similar to that observed with cyclic AMP-in-

TABLE II. Total DNA, RNA, and Protein Contents in "Differentiated" Mouse Neuroblastoma Cells ($NBE^{-}_{(R)}$) in Culture Induced by Noncyclic AMP Agents.[a]

Treatment	DNA (pg/cell)	RNA (pg/cell)	Protein (pg/cell)
Control	19.8 ± 2.7[b]	26 ± 1.3	152 ± 13
SFM	23.8 ± 2.3	65 ± 9.0	180 ± 12
6-thio-guanine	25.4 ± 3.5	79 ± 12.0	346 ± 7.0
5-BrdU	7 ± 1.5	50 ± 3.4	343 ± 34
X ray	62 ± 13.8	153 ± 27.0	768 ± 39

[a] Cells ($0.5–1 \times 10^6$) were placed in large Falcon plastic flasks, and serum-free medium (SFM), 5-bromodeoxyuridine (5-BrdU, 5 μM), 6-thioguanine (0.5 μM) and X ray (1200 rads) were given separately 24 hr later. The total DNA, RNA, and protein contents were analyzed 3 days after treatment. Each value represents an average of 5–8 samples.

[b] SD.

duced differentiated cells (8). But the DNA content per cell was less than that expected if the diploid cells were arrested in the G_1 phase of the cell cycle. However, it should be mentioned that the neuroblastoma cells are aneuploids (20), and therefore the DNA value in each phase of the cycle may not be comparable to diploid cells.

The X ray-induced "differentiated" cells have threefold higher DNA content than that of controls. This value is much higher than that expected if all cells were accumulated in the G_2 phase of the cell cycle. Since the formation of polyploid cells is a well-established response of irradiated mammalian cell culture, it is suggested that the expression of differentiated phenotype can occur in polyploid cells as well as in G_2 cells.

Our data show that the differentiated phenotype can be expressed in the G_1-, and G_2-, and polyploid cells. The fact that certain mammalian neurons such as Purkinje cells have tetraploid DNA content (21–23) indicates that such mechanisms are also operative *in vivo*.

A marked increase in RNA and protein contents occurred in morphologically "differentiated" cells, which is consistent with the fact that the size of soma and nucleus increases during differentiation. The values of RNA and protein contents in the "differentiated" cells represent an underestimation of real value, because the axon-like processes are lost during Viokase treatment and centrifugation.

Some biochemical features of morphologically "differentiated" cells induced by cyclic AMP differ from those induced by noncyclic AMP agents, whereas others are similar. For example, dibutyryl cyclic AMP (24, 25) increases the tyrosine hydroxylase activity, whereas SFM (25, 26) and X ray (24) do not. X ray (27), 5-BrdU, and 6-thioguanine (unpublished observation) increase the level of catechol-*o*-methyltransferase, whereas dibutyryl cyclic AMP does not (27). Choline acetyltransferase (28) and acetylcholinesterase (10) levels increase in "differentiated" cells induced by cyclic AMP and noncyclic AMP agents. Thus it appears that cyclic AMP induces many differentiated functions in neuroblastoma cells, some of which can be expressed without any change in the intracellular level of cyclic AMP.

Summary. The levels of cyclic AMP, DNA, RNA, and protein were measured in morphologically "differentiated" cells induced by noncyclic AMP agents such as 5-bromodeoxyuridine (5-BrdU), 6-thioguanine, serum-free medium (SFM) and X rays. The cyclic AMP levels in 5-BrdU-, SFM-treated cells increased by about twofold, whereas it remained unchanged in X irradiated and 6-thioguanine-treated cells. This shows that X ray and 6-thioguanine probably bypass the step involving the elevation of cyclic AMP. Sodium butyrate increased the cyclic AMP level without causing morphological differentiation, indicating that an elevation of cyclic AMP is not sufficient for the expression of differentiated phenotype. The RNA and protein contents of "differentiated" cells markedly increased. The DNA content of SFM- and 6-thioguanine-treated cells did not significantly change when compared to controls, indicating that the relative distribution of cells throughout the cell cycle was about the same as controls. The DNA content of 5-BrdU treated cells markedly decreased, indicating that most of the cells were accumulated in the G_1 phase. The DNA content of X irradiated cells markedly increased, indi-

cating that the expression of differentiated phenotype occurred in the G_2 phase and/or in the polyploid cells.

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