Neuroblastoma Cell Culture: Membrane Changes During Cyclic AMP-Induced Morphological Differentiation[1] (36750)

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Cultured mammalian cells transformed by the DNA tumor viruses (SV 40 and polyoma) and the RNA tumor viruses (Rous sarcoma virus) agglutinate in the presence of glycoproteins of plant origin, whereas normal contact-inhibited cells do not (1–4). When normal cells are treated briefly with low concentrations of trypsin, agglutinability is increased (5). In addition, normal cells expose the receptor sites during mitosis without any prior trypsin treatment (6). Dibutyryl cyclic AMP decreases the agglutinability of Chinese hamster ovary cells (7) and polyoma-transformed and spontaneous-transformed mouse fibroblasts (8). Agents (dibutyryl cyclic AMP, prostaglandins and inhibitors of cyclic nucleotide phosphodiesterase) which increase the endogenous cellular level of cyclic AMP, cause morphological differentiation of neuroblastoma cells in culture (9–11). Mouse neuroblastoma cells grown in culture maintain the characteristic of unregulated growth, whereas cyclic AMP-induced differentiated cells exhibit the features of differentiated neurons (9–11). Therefore, it was thought that neuroblastoma cells would agglutinate in the presence of plant glycoproteins, whereas the differentiated cells would not. This study shows that control neuroblastoma cells agglutinate in the presence of concanavalin A (Con A) and wheat germ agglutinin (WGA), whereas the morphologically differentiated cells induced by an inhibitor of cyclic nucleotide phosphodiesterase do not. Another study using dibutyryl cyclic AMP and prostaglandin E_1 suggests, however, that these membrane changes are not necessarily linked with the morphological differentiation or the growth rate of this neuronal cell line.

Material and Methods. The procedure for culturing neuroblastoma cells and some morphological features have been previously described (12, 13). In brief, cells are grown in Falcon plastic flasks or dishes containing F12 medium with 10% agamma globulin, newborn calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml); and are maintained at 36° in a humidified atmosphere of 5% CO_2 in air. Neuroblastoma clone NBA 2(1) was used in this study. This clone is an adrenergic clone because it has tyrosine hydroxylase (14) but no choline acetyltransferase (Prasad, Mandal, Waymire, Lee, Vernadakis and Weiner, unpublished data). This cell line has acetylcholinesterase but no butyrylcholenesterase. 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO 20-1724), an inhibitor of cyclic nucleotide phosphodiesterase (15), $N^6O'_2$-dibutyryl-adenosine-3′,5′-cyclic monophosphate (dibutyryl cyclic AMP) and prostaglandin E_1 (PGE_1) were used to cause morphological differentiation of neuroblastoma cells (9–11). RO 20-1724 and PGE_1 were dissolved separately in 95% ethyl alcohol and dibutyryl cyclic AMP was dissolved in F12 medium without serum. All solutions were kept in the freezer until used. Neuroblastoma cells were treated with RO 20-1724 (200 μg/ml, dibutyryl cyclic AMP (50 μg/ml) or pros-

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TABLE I. Agglutinability of Neuroblastoma Cells in Culture.[a]

Treatment	Days after treatment	WGA (250 μg/ml)	Con A (250 μg/ml)
Control	1–4	4+	4+
RO 20-1724 (200 μg/ml)	1	4+	4+
	2	1+	3+
	3	0	1+
	4	0	0
Dibutyryl cyclic AMP (50 μg/ml)	4	4+	4+
Prostaglandin E_1 (10 μg/ml)	4	4+	4+

[a] Neuroblastoma cells (0.5×10^6) were plated in the large Falcon plastic flask and dibutyryl cyclic AMP, prostaglandin E_1 and 4-(-3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO 20-1724) were added separately 24 hr later. Fresh growth medium and drug solutions were added 2 days after treatment because of rapid change in the pH of the medium. Each experiment was repeated at least three times.

taglandin E_1 (10 μg/ml) 24 hr after plating. Control cultures received an equal volume of solvent. Wheat germ agglutinin (WGA) was prepared by Burger and Goldberg's method (2) and concanavalin A (Con A) was purchased from Calbiochem.

The agglutination assay was done 1, 2, 3, and 4 days after treatment with RO 20-1724 and 4 days after dibutyryl cyclic AMP and PGE_1 treatment. The cells were washed 3× with PBS, then 3× with PBS and EDTA (0.5 m*M*) and incubated at 37° in the PBS-EDTA solution until the cells were rounded and detached from the surface. Loosely attached cells were removed by gentle aspiration with a Pasteur pipette. The cells were centrifuged for 3 min at 500 rpm and washed twice with isotonic saline. A cell suspension at a density of 10^6 cells/ml were used for the assay. For each assay, 0.1 ml of cells were used and WGA (125 μg/ml) or Con A (250 μg/ml) was added. To study the effect of trypsin treatment, cells were incubated in the presence of 0.05% trypsin for 5 min at room temperature and then an equal amount of trypsin inhibitor was added. After washing with saline, the cells were examined for agglutination. The cells were examined in a hanging drop on an inverted depression slide after 10–15 min incubation at room temperature.

Agglutination was scored from 0 to 4+, depending on the percentage of cells in clumps and the number of cells in a clump. A 1 + had 50% of the cells agglutinated with 2–3 cells/clump; 2+ had 75% of the cells clumped with 2–10 cells/clump; 3+ had 90% of the cells in clumps of 5–10 cells; and 4+ had greater than 95% of the cells agglutinated with 10–20 cells/clump.

The specificity of the reaction was determined by inhibition with the specific sugar hapten of the individual lectins. Mannose (2.5 m*M*) and *N*-acetylglucosamine (2.5 m*M*) were used as inhibitors of Con A and WGA, respectively.

Results and Discussion. Untreated neuroblastoma cells obtained from exponential phase of growth agglutinated in the presence of Con A (125 μg/ml) and WGA (250 μg/ml). *N*-Acetylglucosamine inhibited the agglutination with WGA, whereas mannose inhibited the agglutination with Con A. The inhibitor of cyclic nucleotide phosphodiesterase (RO 20-1724) causes irreversible morphological differentiation of neuroblastoma cells (11). Table I shows that neuroblastoma cells 4 days after treatment with RO 20-1724 did not agglutinate in the presence of either WGA or Con A. This indicates that the morphologically differentiated cells have the agglutinin receptor sites in a cryptic posi-

tion. It is further shown that the WGA receptor sites seem to become inaccessible earlier than Con A receptor sites. Two days after RO20-1724 treatment, the cells are still sensitive to Con A (3+) but much less (1+) to WGA agglutination. Trypsin treatment renders the RO20-1724-treated cells more agglutinable to Con A than WGA. Indeed, after exposure (5 min at room temperature) to trypsin (50 μg/ml) the differentiated neuroblastoma cells (4 days after treatment with cyclic nucleotide phosphodiesterase inhibitor) did not agglutinate in the presence of WGA, but gave a 2–3+ reaction with Con A. The above data indicate that trypsin treatment unmasks only a portion of the membrane (*i.e.*, that part associated with the Con A receptor sites), whereas WGA receptor sites remained cryptic. This is in contrast to other normal cell lines in which agglutinability to both Con A and WGA is increased by trypsin treatment (5). It has been postulated that increased agglutinability is essential for the renewal of cell division (5), but the differentiated neuroblastoma cells do not resume cell division after trypsin treatment. This is indicated by the fact that when trypsin-treated differentiated neuroblastoma cells (10^5) were plated in the Falcon plastic dishes, the cell number 4 days later was similar to those which were not treated with trypsin. Thus, trypsin treatment, which caused increased agglutinability with Con A, neither caused increased agglutinability with WGA nor led to renewal of growth in cells differentiated by the inhibitor of cyclic nucleotide phosphodiesterase. These results suggest that neuroblastoma system is not similar to the fibroblasts usually studied.

A further study using other agents to induce morphological differentiation reveals that above membrane changes are not always associated with the morphological differentiation of neuroblastoma cells. For example, dibutyryl cyclic AMP and prostaglandin E_1 induced differentiated neuroblastoma cells agglutinated (4+) in the presence of Con A and WGA. Thus, the membrane changes associated with the morphological differentiation induced by inhibition of cyclic AMP phosphodiesterase is different from those by dibutyryl cyclic AMP and prostaglandin E_1, because the former renders the cells unagglutinable, whereas the latter agents do not, although all of the above agents produce similar morphological changes and inhibition of growth. The present study indicates that changes in the agglutinin sites are not necessarily linked with the morphological differentiation or the growth of neuroblastoma cells.

Summary. Mouse neuroblastoma cells agglutinated in the presence of glycoprotein of plant origin (Con A and wheat germ agglutinin), whereas morphologically differentiated cells induced by 4-(3-butoxy-4-meth oxybenzyl)-2-imidazolidinone (inhibitor of cyclic nucleotide phosphodiesterase) did not. WGA agglutinability is lost earlier than Con A during morphological differentiation of neuroblastoma cells. Trypsin treatment unmasked only a portion of Con A agglutinin receptor sites but did not unmask WGA receptor sites at all. The differentiated cells for the most part did not renew cell division after being treated with trypsin. Dibutyryl cyclic AMP or prostaglandin E_1-induced differentiated cells showed agglutination in the presence of Con A and WGA. Thus, it was concluded that changes in the agglutinin sites are not necessarily linked with the morphological differentiation or the growth of neuroblastoma cells in culture.

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