ALTERED ENZYME EXPRESSION IN "DIFFERENTIATED" MURINE NEUROBLASTOMA CELLS

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Summary.—Out of 17 enzymes studied, only 9 were detectable by starch gel electrophoresis in mouse neuroblastoma cells in culture. Prostaglandin E1 (PGE1) and 4(-3-butoxy-4-methoxybenzyl)-2-imidazolidinone (R020-1724), a specific inhibitor of cAMP phosphodiesterase, were used to induce "differentiation". Lactate and 6-phosphogluconate dehydrogenases and adenylate kinase were expressed as single bands in untreated neuroblastoma and induced "differentiated" cells, but the electrophoretic mobility of these enzymes in PGE1-treated cells was slower than that in malignant and R020-1724-treated cells. Three bands of glucose 6-phosphate dehydrogenase were detectable in PGE1-treated cells, whereas the R020-1724-treated cells had two bands and the untreated neuroblastoma cells had only one band. Aldolase was also expressed as a single band; however, the activity of this enzyme was much higher in PGE1-treated cells, whereas the activity was barely detectable for R020-1724-treated and untreated neuroblastoma cells. Some of the enzymes which are present in vivo are absent in vitro. Alkaline phosphatase is present in brain but is absent in neuroblastoma cells in vivo and in vitro. Two bands each of triose phosphate isomerase, fumarase and aldolase are present in brain, but only one band of these enzymes is present in neuroblastoma cells. Although PGE1 and R020-1724 induce many differentiated functions in neuroblastoma cells in a similar manner, PGE1 appears to change characteristically the expression of several enzymes.

AN ELEVATION of the intracellular level of cAMP in neuroblastoma cells in culture induces many differentiated functions which are characteristic of mature neurons. These include formation of long neurites, (Prasad and Hsie, 1971) increase in size of soma and nucleus associated with an increase in total RNA (Augusti-Tocco et al., 1973; Prasad et al., 1973) blockade of cells in G1-phase of cell cycle, (Prasad et al., 1973) increase in activities of tyrosine hydroxylase, (Richelson, 1973; Waymire, Weiner and Prasad, 1972) choline acetyltransferase (Prasad and Mandal, 1973) and acetylcholinesterase, (Furmanski, Silverman and Lubin, 1971; Blume et al., 1970), loss of tumourigenicity (Prasad, 1972) and increase in sensitivity of adenylate cyclase to catecholamines (Prasad and Kumar, 1974). We have shown that the muscle-type lactate dehydrogenase (LDH-5) which prevails in embryonic tissue (Wolf and Engel, 1972) is present in neuroblastoma cells, but absent from mouse brain tissue (Prasad, Prasad and Prasad, 1973), indicating the re-expression of an embryonic feature during malignant transformation. In addition, it has been reported (Bondy, Prasad and Purdy, 1974) that the amount of poly-adenylc-(A)-containing cytoplasmic RNA is greater in cAMP-induced "differentiated" neuroblastoma cells than that in malignant cells. These data indicate that there may be an alteration in the expression of genetic information during the
time of "differentiation" of neuroblastoma cells in culture. Therefore, we have investigated the activities of 17 enzymes in malignant and "differentiated" cells. We now report the following: (a) several of the enzymes which are present in brain are absent in neuroblastoma cells in vivo and in vitro; (b) prostaglandin (PGE\(_1\)) -induced changes in the expression of enzyme pattern are different from those induced by 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (R020-1724), an inhibitor of cAMP phosphodiesterase.

### MATERIALS AND METHODS

**Cell culture.**—The procedure for culturing mouse neuroblastoma cells has been previously described by Prasad and Hsie (1971). Previously defined neuroblastoma clone NBA\(_2\) (Bondy et al., 1974) was used in this study. Neuroblastoma cells contain tyrosine hydroxylase and acetylcholinesterase activities, but have no choline acetyltransferase activity. The average doubling time is about 18 h. The procedures for making solutions were previously described (Prasad and Hsie, 1971). Prostaglandin \(\text{E}_1\), a stimulator of adenylate cyclase, and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (R020-1724), a specific inhibitor of cAMP phosphodiesterase (Sheppard, Wiggan and Tsien, 1972) induce many differentiated functions in these cells (Prasad and Kumar, 1974). Therefore, these agents were used to induce differentiation in neuroblastoma cells in culture. PGE\(_1\) (10 \(\mu\)g/ml) and R020-1724 (200 \(\mu\)g/ml) were added separately 24 h after plating the cells (5 \(\times\) 10\(^6\)) in large Falcon plastic flasks. The drug and medium were changed every day and cells were removed from the flask surface, using Viokase solution, 3 days after treatment. The cells were washed twice with phosphate buffer solution.

To prepare the cell homogenate (in vitro study) the cells were harvested by centrifugation and washed twice with 0.9\% saline. The cells were then resuspended in deionized distilled water to make a concentration of 5 \(\times\) 10\(^6\) cells/ml. The cell membranes were then disrupted by alternate freezing and thawing. The supernatant obtained after the centrifugation at 10,000 \(g\) was used for the electrophoresis.

Tumours were produced in male A/J mice by injecting cells of clones NBA\(_2\) (Bondy et al., 1974). The tumour was allowed to grow for 15 days. Crude tissue extracts (in vivo study) of tumour, brain and kidney were prepared as described by Prasad, Prasad and Tevethia (1972). All the samples were subjected to vertical starch gel electrophoresis. The protein concentration of each of these samples ranged from 1.8 to 2.0 mg/ml. The buffer systems used for different enzymes were the same as previously described (Prasad et al., 1974). Gel slices were stained for the following enzymes by the routine methods (Shaw and Prasad, 1970): dehydrogenases of lactate (LDH), malate (MDH), glutamate (GDH), glucose-6-phosphate (G6PD), 6-phosphogluconate (6PGD), hexose-6-phosphate (H6PD), and isocitrate (IDH), \(\alpha\)-esterase, fumarase, phosphoglucomutase (PGM), aldolase, adenylate kinase (AK), acid and alkaline phosphatases, hexokinase (HK) and triose-phosphate isomerase (TPI).

### RESULTS AND DISCUSSION

**In vitro system.**

The Table summarizes the results of our study. Out of 17 enzymes studied, only 9 were detectable by starch gel electrophoresis in neuroblastoma cells in culture. Five of these 9 enzymes showed electrophoretic variation. LDH and 6PGD and AK were expressed as a single band in malignant cAMP-induced and "differentiated" cells, but the electrophoretic mobility of these enzymes in PGE\(_1\)-treated cells was slower than in control (untreated neuroblastoma) cells, R020-1724-treated cells and tumour tissue extract. Fig. 1 shows the electrophoretic pattern of 6PGD. The pattern of G6PD was interesting. Three bands of this enzyme were detectable in PGE\(_1\)-treated cells whereas the R020-1724-treated cells had two bands. The control cells as well as tumour tissue extract had only one band (Fig. 2). Aldolase was also expressed as a single band; the activity was much higher in PGE\(_1\)-treated cells, whereas the activity was barely detectable for R020-1724-treated and control cells.


**Table.**—Expression of Enzymes of PGE$_1$- and R020-1724-treated Murine Neuroblastoma Cells in Culture and in Brain and Tumour Tissues

| No. | Enzyme              | In vitro system | In vivo system |
|-----|---------------------|-----------------|---------------|
|     | R020-1724-            | Control          | Presence of electro-| Presence of electro-|
|     | treated              | treated         | phoretic variants | phoretic variants |
|     | cells                | cells           | Tumour | Brain | Kidney | Tumour | Brain | Kidney |
| 1   | IDH                  | —               | —      |       | N.D.   | —      |       | N.D.   |
| 2   | Alkaline phosphatase | —               | —      |       |        | —      |       | N.D.   |
| 3   | GPD                  | —               | —      |       |        | —      |       | N.D.   |
| 4   | GDH                  | +               | +      |       | +      | +      | +      | +      |
| 5   | α-esterase           | —               | —      |       |        | —      |       | —      |
| 6   | HK                   | —               | —      |       |        | —      |       | —      |
| 7   | H6PD                 | —               | —      |       |        | —      |       | —      |
| 8   | Acid phosphatase     | +               | +      |       | +      | +      | +      | +      |
| 9   | Aldolase             | +               | +      |       | +      | +      | +      | +      |
| 10  | G6PD                 | +               | +      |       | +      | +      | +      | +      |
| 11  | 6PGD                 | +               | +      |       | +      | +      | +      | +      |
| 12  | TPI                  | +               | +      |       | +      | +      | +      | +      |

| Enzyme              | Presence             | Tumour | Brain | Kidney |
|---------------------|----------------------|--------|-------|--------|
| Isocitrate dehydrogenase | —                  | —      |       | N.D.   |
| α-GPD               | Lactate dehydrogenase| —      | —      | —      |
| Glutamate dehydrogenase | —                  | —      | —      | —      |
| Hexokinase          | Glucose-6-phosphate dehydrogenase | — | — | — |
| Hexose-6-phosphate dehydrogenase | — | — | — | — |
| Phosphoglucomutase  | Triose phosphate isomerase | — | — | — |
| Adenylate kinase    | Present              | —      | —      | —      |
| Malate dehydrogenase | Not done            | —      | —      | —      |

**Fig. 1.**—Starch gel electrophoretic patterns of 6PGD from differentiated neuroblastoma cells in vitro (o, origin). Samples are: (a) tumour tissue extract, (b) control cells, (c) R020-1724-treated cells, (d) PGE$_1$-treated cells.

**Fig. 2.**—Starch gel electrophoretic patterns of 6PGD from differentiated neuroblastoma cells in vitro (o, origin). Samples are: (a) PGE$_1$-treated cells, (b) R020-1724-treated cells, (c) control cells, (d) tumour tissue extract.
In vivo system

HK and H6PD were the only 2 enzymes which were not detectable in any tissue (Table). IDH was present in kidney but absent in brain and tumour tissue. Eight of the enzymes showed electrophoretic variation. A single band of AK, TPI, G6PD, fumarase and 6PGD hydrogenase was present in tumour tissue. In the samples of brain tissue, however, 2 bands of aldolase, fumarase and TPI were present. Two bands of fumarase and TPI were also present in kidney tissue.

The result shows that some of the enzymes which are present in vivo are absent in vitro (Nos. 1–6 of Table). Alkaline phosphate is present in brain and kidney tissue but absent in neuroblastoma cells in vivo and in vitro. We do not know if the absence of enzymes from the tumour tissue reflects an embryonic or a malignant characteristic or the lack of glial components of the nervous tissue. The presence of 2 bands of TPI, fumarase and aldolase in brain, and the presence of single bands of these 3 enzymes in tumours, indicate either the loss of tissue-specific isozyme during malignant transformation, or that the 2nd bands of these enzymes are of glial origin and therefore may be demonstrated in brain but not in pure neuronal culture. Therefore, any change or lack of it in the expression of the enzyme pattern in "differentiated" cells when compared to brain tissue may or may not be the differentiated functions of mature neurons.

Although PGE$_1$ and R020-1724 induce many differentiated functions in neuroblastoma cells in a similar manner, (Furmanski, Silverman and Lubin, 1971, Prasad and Kumar, 1974) PGE$_1$ appears to change characteristically the expression of AK and G6PD, 6PGD and LDH. From these data it is clear that PGE$_1$-treated neuroblastoma cells in culture express different enzyme patterns from those cells which were treated with R020-1724. Previous studies have shown that many differentiated functions in neuroblastoma cells are induced both by PGE$_1$ and R020-1724 (Furmanski et al., 1971; Prasad and Kumar, 1974). However, these agents produce different types of membrane changes (Prasad and Sheppard, 1972). For example, R020-1724-induced "differentiated" neuroblastoma cells do not agglutinate, whereas PGE$_1$-induced "differentiated" cells agglutinate similarly to malignant cells. Our present study shows that certain enzymatic expressions of PGE$_1$-treated cells are also different from those of R020-1724-treated cells. These data suggest that the expression of enzyme patterns in PGE$_1$-treated cells is different from that of R020-1724-treated cells, although both agents increase intracellular levels of cAMP and induce many differentiated functions in a similar manner (Furmanski et al., 1971; Gilman and Nirenberg, 1971; Richelson, 1973).

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