Adrenergic,¹ Cholinergic, and Inactive Human Neuroblastoma Cell Lines with the Action-Potential Na⁺ Ionophore²

Gregory J. West, Jiro Uki, Harvey R. Herschman,³ and Robert C. Seeger⁴

Department of Biological Chemistry and Laboratory of Nuclear Medicine and Radiation Biology [G. J. W., J. U., H. R. H.], and the Department of Pediatrics [R. C. S.], UCLA School of Medicine, Los Angeles, California 90024

SUMMARY

Cultured human neuroblastoma cell lines were assayed for biochemical characteristics of neuronal function. Cell lines studied included LA-N-1, LA-N-2, IMR-32, SK-N-SH, and SK-N-MC. Veratridine-dependent uptake of ²²Na⁺ implied the presence of the action potential Na⁺ ionophore in LA-N-1, LA-N-2, IMR-32, and SK-N-SH. The time course of ²²Na⁺ uptake and inhibition of uptake by tetrodotoxin supported this. SK-N-MC had no veratridine-dependent ²²Na⁺ uptake. Tyrosine hydroxylase (EC 1.14.10.), glutamic acid decarboxylase (EC 4.1.1.15), and acetylcholine contents in neuroblastoma cells were compared to those in brain. LA-N-1 and IMR-32 contained 15 and 5 times as much tyrosine hydroxylase, respectively, whereas LA-N-2, SK-N-SH, and SK-N-MC contained only 0.5 to 5% of that in brain. Acetylcholine was present in LA-N-2 in 15- to 20-fold greater quantities than in brain; other lines had only 10 to 50% of that in brain. None of the cell lines contained glutamic acid decarboxylase. Thus, continuously propagated human neuroblastoma cell lines may have the action potential Na⁺ ionophore and may be adrenergic (LA-N-1 and IMR-32), cholinergic (LA-N-2), or inactive (SK-N-SH and SK-N-MC). This is the first demonstration of the action potential Na⁺ ionophore and of acetylcholine production in human neuroblastoma cell lines.

INTRODUCTION

Established neuroblastoma cell lines provide models for studying both neoplasia and normal differentiation and function of neurons (11). Murine C1300 neuroblastoma cells growing in mice have been used as a laboratory model to develop chemotherapy for human neuroblastoma (9). In vitro C1300 cells synthesize neurotransmitters (3), have the action potential Na⁺ ionophore (7), and can have brain-specific cell-surface antigens (1, 2). Furthermore, morphological differentiation of these cells in vitro is accompanied by alterations in neurochemical, electrophysiological, and cell-surface properties. These studies suggest that understanding of human neuroblastoma and possibly of neuronal differentiation and function could be advanced if a number of well-characterized human neuroblastoma cell lines were available for analysis of both tumor-associated and organ-specific characteristics.

Two new human neuroblastoma cell lines were recently established in 1 of our laboratories (22). These lines along with 3 previously established lines (4, 24) have been examined for biochemical properties of neural tissues. We report that continuous lines of human neuroblastoma cells possess the action potential Na⁺ ionophore and can be adrenergic, inactive, or cholinergic.

MATERIALS AND METHODS

Reagents and Supplies. Reagents and their sources are: L-[3,5-³H]tyrosine (1.0 Ci/mmmole), ²²NaCl (carrier free), and DL-[¹-¹⁴C]glutamic acid (23 mCi/mmmole) from Amersham/Searle, Arlington Heights, Ill.; Liquifluor from New England Nuclear, Boston, Mass.; tetrodotoxin (purified by Sankyo), catalase, pyridoxal phosphate, and DL-6-methyl-5,6,7,8-tetrahydropterine dihydrate from Calbiochem, La Jolla, Calif.; hydroxide of Hyamine 10-X from Packard Instrument Co., Downers Grove, Ill.; tyrosine and ouabain from Sigma Chemical Co., St. Louis, Mo.; Hi-Pure nitrogen from Liquid Carbonic Corp., Los Angeles, Calif.; CGC-241 and Dowex 50-2X from J. T. Baker Chemical Co., Phillipsburg, N. J.; and veratridine from Aldrich Chemical Co., Milwaukee, Wis. All other reagents were reagent grade. Plastic wells ("center wells") were obtained from Kontes Glass Co., Vineland, N. J.

Cell Culture. Cell culture is described in detail in the preceding paper (22). Briefly, LA-N-1 was derived from bone marrow metastases of a neuroblastoma in a 2-year-old male. LA-N-2 was derived from a biopsy specimen of the primary abdominal neuroblastoma of a 3-year-old female (21). Both lines were grown in Waymouth's medium containing 30% heat-inactivated fetal calf serum, glutamine (100 mM), and gentamycin (50 µg/ml). SK-N-SH (4), SK-N-MC (4), IMR-32 (24), T24 (6), MT (14), and J82 (18) were grown in Eagle's minimal essential medium containing 15% heat-inactivated fetal calf serum, glutamine (100 mM), and gentamycin (50 µg/ml).
Veratridine-dependent Na\(^+\) Uptake. Cell lines were assayed for veratridine-dependent Na\(^+\) uptake according to the procedure of Catterall and Nirenberg (7). Cells grown to a confluent monolayer in 60-mm culture dishes were preincubated at 37\(^\circ\)C for 2 to 3 min in a shallow water bath with 2 ml of assay medium (50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 130 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 0.8 mM MgSO\(_4\), 5.5 mM glucose, and 1.0 mM Na\(_2\)HPO\(_4\) adjusted to pH 7.4). The medium was then aspirated and replaced with assay medium containing 5 mM NaH\(_2\)PO\(_4\) adjusted to pH 7.4. The assay medium and wash medium were modified slightly or for a variable time in the case of the time-course study. The assay medium was aspirated, and the plate was washed rapidly 4 times with wash medium (164 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 0.8 mM MgSO\(_4\), and 5.0 mM Na\(_2\)HPO\(_4\) adjusted to pH 7.4). The cell monolayer was dissolved with 2 ml of 0.4 N NaOH. One ml was counted in a gamma counter, and 0.2 ml was used to measure protein concentration (13).

Acetylcholine. Cells were grown to confluence in 100-mm tissue culture dishes, washed 3 times with 0.14 M NaCl, 0.15 mM CaCl\(_2\), 0.50 mM MgSO\(_4\), and 0.01 M 2-amino-2-hydroxy-propanediol base adjusted to pH 7.45 with HCl. The cell monolayer was homogenized in 2 ml of formic acid:acetone (15:85). Particulate matter was removed by centrifugation. After 2 ether washes, acetylcholine was extracted with dipicrylamine. The sample was injected and shaking was continued for another 30 min. At the end of the experiment, the plastic well was placed in 10 ml of Liquifluor:toluene (42:1000) for counting.

Tyrosine Hydroxylase. Tyrosine hydroxylase was measured by a modification of the method of Nagatsu et al. (16). L-[3,5-\(^3\)H]Tyrosine was purified by absorption to and elution from Dowex 50-X2. L-[3,5-\(^3\)H]Tyrosine (250 \(\mu\)l) was acidified with 0.2 ml of 0.17 N acetic acid and absorbed to the resin. The column was washed with 100 ml of distilled water and then with 10 ml of 0.5 N HCl. The radioactive tyrosine was eluted with 20 ml of 3 N HCl. The HCl was evaporated with a rotary evaporator, and the sample was resuspended and stored in absolute ethanol.

Cells (two 100-mm Petri plates) were harvested as follows. Medium was removed from the plates. Cells were washed 3 times with cold 0.9% NaCl solution. To each plate was added 0.1 ml of 1 M potassium phosphate buffer, pH 6.2. Cells were scraped from the dish with a rubber policeman and transferred to a test tube with a Pasteur pipet. Cell homogenates were prepared by freezing and thawing 3 times. The supernatant from a 1000 \(\times\) g, 10-min centrifugation was used for protein and tyrosine hydroxylase determinations.

For assay of enzyme activity, 3 \(\times\) \(10^9\) cpm of L-[3,5-\(^3\)H]tyrosine (for each assay tube) were evaporated in a stream of nitrogen and resuspended in 10 \(\mu\)l of 1 M L-tyrosine per assay tube. The reaction mixture, which was preincubated for 2 min at 37\(^\circ\)C, contained 0.1 M potassium phosphate buffer (pH 6.2), 2000 units of catalase, 1 M DL-6-methyl-5,6,7,8-tetrahydropteridine dihydroxide, 0.1 mM mercaptoethanol, and enzyme solution in a final volume of 100 \(\mu\)l. The reaction was started by the addition of 10 \(\mu\)l of the tyrosine solution. After 20 min at 37\(^\circ\), 200 \(\mu\)l of 0.17 M acetic acid were used to stop the reaction. A boiled enzyme solution was used to determine the blank. Tritiated water was isolated by chromatography over Dowex 50-X2 and counted in 10 ml of Bray’s solution (5). All assays were done in duplicate. We found the monomethyl derivative of 5,6,7,8-tetrahydropteridine to give 2.5 times the tyrosine hydroxylase activity as that observed with the 6,7-dimethyl derivative.

RESULTS

Veratridine-dependent Na\(^+\) Uptake. Veratridine-dependent Na\(^+\) uptake implies the presence of the action potential Na\(^+\) ionophore (7). Neuroblastoma cell lines LA-N-1, LA-N-2, SK-N-SH, and IMR-32 each have a large veratridine-dependent Na\(^+\) uptake (Table 1). In contrast, line SK-N-MC consistently had small positive values of veratridine-dependent Na\(^+\) uptake which were not significantly different from control human cell lines T24, derived from a bladder carcinoma, and MT, derived from an osteogenic sarcoma.
Tetrodotoxin was used to characterize further the nature of the veratridine-dependent Na⁺ uptake. Veratridine-dependent Na⁺ uptake was measured in the presence of 0.001, 0.1, and 10 μM tetrodotoxin (Table 1). Uptake of Na⁺ by LA-N-1, LA-N-2, SK-N-SH, and IMR-32 was inhibited by tetrodotoxin. The concentration resulting in 50% inhibition ranged from 0.03 to 0.8 μM for the various cell lines.

If veratridine-dependent Na⁺ uptake represents (a) passive diffusion of Na⁺ through the open Na⁺ ionophore with (b) subsaturating Na⁺ concentration, then the time course for uptake of Na⁺ should be given by a single exponential and should fit the equation (7):

$$\ln \left( \frac{[\text{Na}^+]_{in}}{[\text{Na}^+]_{in}} \right) = kt$$

(A)

where $\frac{[\text{Na}^+]_{in}}{[\text{Na}^+]_{in}}$ is the concentration of radioactivity inside the cell at equilibrium and $\frac{[\text{Na}^+]_{in}}{[\text{Na}^+]_{in}}$ is the concentration at time t. If the left side of the equation is plotted against time, a straight line with slope k is the result. SK-N-SH was chosen as a representative cell line and assayed (Chart 1). It is apparent from the straight-line plot that Equation A is satisfied, consistent with both assumptions.

Neurotransmitters. Cultured neuroblastoma cell lines were assayed for tyrosine hydroxylase, glutamic acid decarboxylase, and acetylcholine (Table 2). Activities in cell lines were compared to activities found in freshly prepared mouse or rat brain. LA-N-1 and IMR-32 contained 15 and 5 times as much tyrosine hydroxylase, respectively, as brain extracts. In contrast, LA-N-2, SK-N-SH, and SK-N-MC contained only 0.5 to 5% of the tyrosine hydroxylase activity of brain. Acetylcholine was present in LA-N-2 in 20-fold greater quantities than in brain; other lines had only 10 to 50% that in brain. None of the cell lines had high specific activities of glutamic acid decarboxylase when compared to brain or nonneuronal cell lines.

Clinical Correlations. All tumors were diagnosed pathologically as neuroblastomas; however, not all produced catecholamines. The patient from whom LA-N-i was derived had elevated levels of vanillylmandelic and homovanillic acid in his urine, and the cell line had high levels of tyrosine hydroxylase. LA-N-2 was derived from a child who did not excrete catecholamines, and the cell line lacked tyrosine hydroxylase. However, a freshly frozen sample of the tumor from which LA-N-2 was derived contained acetylcholine as did the cell line. Line SN-N-MC lacked tyrosine hydroxylase; the patient did not excrete catecholamines (4). Urinary catecholamines were not reported for the patient from whom IMR-32 was derived. The only discrepancy was with SK-N-SH. In this case the patient excreted vanillylmandelic acid, but tyrosine hydroxylase was not present in the cell line. However, Biedler et al. (4) reported that SK-N-SH cells have dopamine-β-hydroxylase, an enzyme characteristic of adrenergic cells.

DISCUSSION

Human neuroblastoma cells in continuous culture were found to retain membrane transport and neurotransmitter functions characteristic of nervous tissue. Using these functions, the cell lines were classified as positive or negative for the action potential Na⁺ ionophore and as adrenergic, inactive, or cholinergic.

Veratridine-dependent Na⁺ uptake implies that a cell line has the action potential Na⁺ ionophore and thus is likely to be electrophysiologically active. Human neuroblastoma cell lines have not been examined previously for this property. Four of the 5 lines tested, LA-N-1, LA-N-2, SK-N-SH, and IMR-32, had veratridine-dependent Na⁺ uptake. This observation was extended by using tetrodotoxin, a specific inhibitor of the action potential Na⁺ ionophore (17), to inhibit veratridine-dependent Na⁺ uptake. The equilibrium constant for binding of tetrodotoxin to nerve prepara-
Neurotransmitters and enzymes of neurotransmitter biosynthesis in human cell lines

Specific activities of tyrosine hydroxylase and glutamic acid decarboxylase are reported. One unit of tyrosine hydroxylase activity is equal to 1 pmole of product per min. One unit of glutamic acid decarboxylase activity is equal to 1 pmole of product per min.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tyrosine hydroxylase (units/mg protein)</th>
<th>Acetylcholine (nmol/g tissue)</th>
<th>Glutamic acid decarboxylase (mmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA-N-1</td>
<td>668 ± 17a</td>
<td>10.8 ± 7.8</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>LA-N-2</td>
<td>0.0b</td>
<td>504 ± 209</td>
<td>0.0 ± 0.05</td>
</tr>
<tr>
<td>SK-N-MC</td>
<td>0.5 ± 0.6</td>
<td>7.8 ± 4.9</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>SK-N-SH</td>
<td>5.3 ± 0.7</td>
<td>4.2 ± 3.5</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td>IMR-32</td>
<td>219 ± 12</td>
<td>2.5 ± 1.0</td>
<td>0.1 ± 0.04</td>
</tr>
<tr>
<td>T24</td>
<td>0.7 ± 1.5</td>
<td>1.4 ± 1.0</td>
<td>0.0 ± 0.01</td>
</tr>
<tr>
<td>MT</td>
<td>0.3 ± 0.6</td>
<td>0.9 ± 1.0</td>
<td>0.0 ± 0.05</td>
</tr>
<tr>
<td>J82</td>
<td>2.1 ± 0.2</td>
<td>1.5 ± 0.5</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Brain</td>
<td>41.2 ± 1.1c</td>
<td>22c</td>
<td>5.3 ± 1.5c</td>
</tr>
</tbody>
</table>

*a* Mean ± S.D.
*b* Values less than blank.
*c* Rat.
*d* Mouse.

Table 2

Neurotransmitters and enzymes of neurotransmitter biosynthesis in human cell lines

Our finding that IMR-32 cells have elevated levels of tyrosine hydroxylase differs from the report of Prasad et al. (19) who found that IMR-32 cells had no detectable tyrosine hydroxylase activity. However, they did induce 9 pmoles/min/mg protein by 5-bromodeoxyuridine treatment. Aside from possible differences in culture conditions or divergence of cell lines, we cannot account for this difference.

LA-N-2 is the 1st human neuroblastoma cell line demonstrated to produce acetylcholine. A cholinergic clone of murine neuroblastoma C1300 has been reported (3); however, LA-N-2, even before cloning, appears to be synthesizing acetylcholine more actively. Three additional human neuroblastoma cell lines recently have been reported to have choline acetyltransferase, another marker for cholinergic activity (20); however, 2 lines also had tyrosine hydroxylase, leaving one that expressed only the cholinergic marker. None of these lines appear to be as active as LA-N-2 since choline acetyltransferase levels ranged from 200 to 495 pmoles/min/mg protein. Control values for choline acetyltransferase in brain and nonneural tissues were not included in this report; however, Wilson et al. (25), using the same method, reported mouse brain to have 1500 pmoles/min/mg protein. The discovery that LA-N-2 produced only acetylcholine and that the biopsy specimen from which LA-N-2 was derived also had acetylcholine may have significant clinical implications, both for differential diagnosis of neuroblastoma-like tumors that do not produce catecholamines and for estimating prognosis.

LA-N-1 is the 1st human neuroblastoma cell line demonstrated to have the action potential Na⁺ ionophore, tyrosine hydroxylase, and cytoplasmic dense cores (22) in combination, suggesting that these cells have the capability of synthesizing and packaging catecholamines. If this is true, and if the cells can be induced to release catecholamines, LA-N-1 may provide a good human-derived model for many studies of adrenergic neurons.

LA-N-2, together with LA-N-1, provides a valuable model for comparing cholinergic and adrenergic cells. It has not been possible to obtain completely pure populations of neurons committed to that transmitter, and enzymes involved in its synthesis, significantly greater than that for whole brain since the population of neurons committed to that transmitter will account for only a fraction of the mass of brain.

Cell lines LA-N-1 and IMR-32 contained large amounts of tyrosine hydroxylase relative to brain. We therefore conclude they are adrenergic. In contrast, LA-N-2 had large amounts of acetylcholine, and we conclude it is cholinergic. SK-N-SH and SK-N-MC lacked tyrosine hydroxylase and acetylcholine, and by these criteria we classify them as inactive. However, SK-N-SH has been reported to have dopamine β hydroxylase, an adrenergic marker, and SK-N-MC has choline acetyl transferase, a cholinergic marker (Ref. 4; J. Biedler, personal communication). The discordance between these latter markers and those we utilized suggests that neuroblastoma cells can express markers of a given differentiation pathway to variable degrees. This is supported by our observation that LA-N-1 cells but not those of the other adrenergic cell lines have multiple cytoplasmic dense cores (22). Our findings, together with those of Biedler et al. (4), are consistent with the clinical histories suggesting that the established cell lines are representative of the tumors from which they were derived.

Clinical implications, both for differential diagnosis of neurolblastoma-like tumors that do not produce catecholamines and for estimating prognosis, may provide a good human-derived model for many studies of adrenergic neurons.
neurons, much less pure samples of a particular type of neuron. These cell lines, which originated from single clones (22) and which express either adrenergic or cholinergic properties, offer the opportunity to perform ultrastructural, electrophysiological, and biochemical comparisons on homogeneous populations of cells with neuronal characteristics. In this respect, they complement the various murine clones (3) and the recently described functional rat neural tumors (20). Finally, they also may be uniquely valuable as experimental models for developing new and improved therapies of childhood neuroblastoma.

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