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Selection for Neuroblastoma Cells that Synthesize Certain Transmitters

(differentiation/tyrosine hydroxylase/catecholamines/cell culture/genetics)

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ABSTRACT **A selection procedure was devised for neurons and related cells that depends upon the ability of the cells to synthesize certain amine neurotransmitters. The rationale for selection is that tyrosine is an essential amino acid for most mammalian cells and that three enzymes from mammalian sources can catalyze the synthesis of tyrosine: phenylalanine hydroxylase (EC 1.14.16.1), tyrosine hydroxylase (EC 1.14.16.2), and tryptophan hydroxylase (EC 1.14.16.4). Tyrosine hydroxylase is found predominantly in adrenergic neurons and related cells that synthesize dopamine, norepinephrine, and epinephrine, and tryptophan hydroxylase in cells synthesizing serotonin or melatonin. Only 1 out of 70,000 uncloned mouse neuroblastoma cells grew well in the absence of tyrosine. Approximately 50% of the cell lines obtained by selection had tyrosine hydroxylase activity. This selection procedure thus provides a simple means of obtaining cell lines of neural origin on the basis of their ability to synthesize putative transmitters.**

It seems likely that a universal code has evolved which enables neurons to communicate with one another by sending and receiving information encoded in the form of neurotransmitters. Many steps in the coding of neural information can be studied with neuroblastoma clones currently available; however, more cell lines with additional neural properties are needed. For this reason we examined various ways of selecting cells of neural origin on the basis of their ability to synthesize transmitters.

In this paper we describe a procedure for selecting adrenergic neuroblastoma cells by their ability to grow in medium lacking tyrosine. The selection procedure depends on the following phenomena: (*a*) Tyrosine is an essential amino acid for most mammalian cell types grown *in vitro* (1); (*b*) tyrosine hydroxylase (EC 1.14.16.2; tyrosine 3-monooxygenase) catalyzes the conversion of phenylalanine to tyrosine, as well as the conversion of tyrosine to 3,4-dihydroxyphenylalanine (dopa) (2, 3) (see Fig. 1); and (*c*) tyrosine hydroxylase activity is found predominantly in adrenergic neurons and related chromaffin cells of the adrenal medulla (4). During the course of these studies, we learned that T. Puck has used a similar procedure to select for cells with phenylalanine hydroxylase (EC 1.14.16.1) (personal communication).

MATERIALS AND METHODS

Cell Culture. The following cell lines were used: an uncloned line of mouse neuroblastoma C1300, neuroblastoma clones N-18, NS-20, and N1E-115 (also referred to as N-115) (5), a rat glioma, C-6, derived by Benda *et al.* (6) (from the American Type Culture Collection, no. CCL107), and mouse L-cells B82 (7). Cells were grown in monolayer culture in polystyrene petri dishes, 60 and 100 mm (20 and 54 cm^2, respectively) (Falcon Plastics) at 37° in a humidified atmosphere of 10% CO_2 and 90% air. Cells were grown routinely in either F-14 medium (8) or the Dulbecco–Vogt modification of Eagle's medium with 4.5 g of dextrose per liter and no Na pyruvate (GIBCO, Catalogue no. H-21). Both media contain tyrosine and phenylalanine and both were supplemented with 5% fetal-calf serum (Colorado Serum). Cells were fed at 1- to 5- day intervals and passaged as described (9).

Cells grown in modified Eagle's medium were adapted to F-14 by cultivation in this medium for at least 10 generations prior to selection. When cloning efficiencies were being determined, cells were trypsinized and triturated to achieve a suspension of single cells and then plated at varying concentrations in either F-14, F-14 minus tyrosine, or F-14 minus tyrosine and phenylalanine. When tyrosine alone was omitted, the phenylalanine concentration was increased to 0.12 mM. All media were supplemented with 5% fetal-calf serum dialyzed for 2 days at 3° against 10 volumes of 0.85% (w/v) NaCl (the NaCl solution was changed after 24 hr). Thirty days after they were plated visible colonies (containing >500 cells) were picked with the aid of porcelain penicylinders (Fisher). Cloning efficiencies were determined in plates containing <100 colonies, due to the possibility of cross-feeding. Cell lines originating from selected colonies were further grown in F-14 without tyrosine for 1 month and then in either F-14 or modified Eagle's medium with tyrosine. Cell lines were free of contamination by pleuropneumonia-like organism (PPLO) as determined by culture in plates and broth, and by autoradiography following incubation with [^{3}H]thymidine. For growth rate experiments, cells were fed daily, dissociated by trypsinization, and counted with the aid of a Coulter counter.

Tyrosine Hydroxylase Assay and Chromatographic Identification of Dopa. Cells were grown in modified Eagle's medium

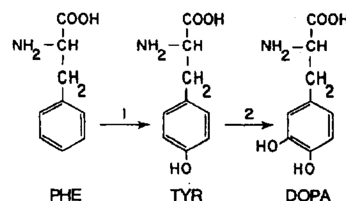

FIG. 1. Reactions catalyzed by tyrosine hydroxylase. Oxygen and tetrahydrobiopterin are required for both reactions as follows: (*1*) L-tyrosine + O_2 + tetrahydropteridine → HOH + L-3,4-dihydroxyphenylalanine + dihydropteridine; (*2*) L-phenylalanine + O_2 + tetrahydropteridine → HOH + L-tyrosine + dihydropteridine.

with 5% fetal-calf serum, maintained in stationary phase for 5–10 days, then harvested, homogenized, and assayed for tyrosine hydroxylase by a modification of the methods of Nagatsu, Levitt, and Udenfriend (10) and Shiman, Akino, and Kaufman (3) as described (5, 11).

Product identification was performed as follows. Reactions contained 0.25 mM *N*-(3-hydroxy-benzyl)-*N*-methylhydrazine (Smith and Nephew Ltd.), an inhibitor of aromatic acid decarboxylase (EC 4.1.1.28) in addition to the usual components. Reactions were deproteinized and neutralized by the method of Udenfriend and Zaltzman-Nirenberg (12). Samples then were passed through alumina oxide columns as described by Nagatsu, Levitt, and Udenfriend (10), and the tritiated reaction product was characterized by thin-layer and paper chromatography using four solvent systems (13), as listed in the *legend* to Table 3.

RESULTS

The Effect of Tyrosine Deprivation on Cell Growth. In this study we have examined the question "Is the growth of neuroblastoma cells with and without tyrosine hydroxylase dependent upon added tyrosine?" The ability of clones N-18 and N-115, without and with tyrosine hydroxylase, respectively, to multiply in complete medium containing both tyrosine and phenylalanine, in medium lacking tyrosine, and in medium lacking both tyrosine and phenylalanine, is shown in Fig. 2. N-18 cells grew well in complete medium, but not in medium deficient in one or both amino acids. In the absence of tyrosine, 50% of these cells died within 8 days. However, N-115 cells with high tyrosine hydroxylase activity [980 pmoles of dopa formed per min/mg of protein (5)] did multiply in medium lacking tyrosine after a lag period. Cell survival was dependent upon phenylalanine, but not upon tyrosine. An N-115 subline obtained by selection in the absence of tyrosine grew well both in the presence and absence of tyrosine with population generation times of 24 and 54 hr, respectively. Again, cells did not survive in the absence of both phenylalanine and tyrosine.

The cloning efficiencies of different cell lines in the presence and absence of tyrosine are shown in Table 1. Neuroblastoma tumor C1300 contains at least three cell types with respect to transmitter synthesis: adrenergic cells, cholinergic cells, and cells with no known transmitter (5). The adrenergic line, N-115, formed colonies with almost equal efficiency in media with or without tyrosine. In contrast, no colonies were observed in medium lacking tyrosine with both a cholinergic clone, NS-20, with high choline acetyltransferase (EC 2.3.1.6), but no tyrosine hydroxylase activity, as well as clone N-18, which lacks both enzyme activities.

The cloning efficiency of an uncloned line of C1300 with little or no tyrosine hydroxylase activity was 16% in the presence of tyrosine. In the absence of tyrosine approximately 1 in 70,000 cells gave rise to a colony. Two other clonal lines, rat glioma C-6 and L-cell clone B82, also did not form colonies in the absence of tyrosine.

When higher concentrations of L-cells were cultivated with neuroblastoma N-115 cells with high tyrosine hydroxylase activity, many colonies were found that contained both neuroblastoma and L-cells; no colonies were found that contained only L-cells. These results suggest that L-cells obtain sufficient tyrosine for growth from the neuroblastoma cells when the two cell types are in close proximity.

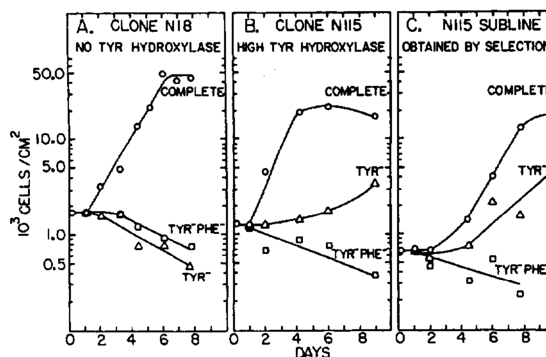

FIG. 2. Growth of neuroblastoma cells in the presence and absence of tyrosine. The growth of neuroblastoma cells with and without tyrosine hydroxylase was determined in the following media: (○) F-14 with tyrosine and phenylalanine (complete); (△) F-14 minus tyrosine with 0.12 mM phenylalanine; and (□) F-14 minus tyrosine and phenylalanine. All media contained 5% dialyzed fetal-calf serum. In *panel A* the growth of neuroblastoma clone N-18 is shown; in *panel B*, clone N-115 with high tyrosine hydroxylase activity; and in *panel C* a subline of clone N-115 (N-T45) derived by selection in medium without tyrosine. Cells were grown in 60-mm Falcon petri dishes (20 cm²) and dissociated and counted as described in *Methods*.

Characterization of Neuroblastoma Cells Selected by Tyrosine Deprivation. Colonies obtained from the uncloned C1300 cell line by selection in medium without tyrosine were further propagated in modified Eagle's medium in the presence of tyrosine. Homogenates were prepared from cells grown to stationary phase and assayed for tyrosine hydroxylase activity. Ten out of the 21 cell lines obtained had tyrosine hydroxylase activity (Table 2). The specific activity of the enzyme varied considerably, as shown.

Homogenates of four of the cell lines and a homogenate prepared from N-115 were used for product characterization.

TABLE 1. *The effect of tyrosine on cell cloning efficiency*

Cell lines		Tyrosine hydroxylase activity	% of cells forming colonies: + Tyrosine	% of cells forming colonies: − Tyrosine
Neuroblastoma C-1300 clones				
Adrenergic	N-115	+	5	1.4
Cholinergic	NS-20	−	9	<0.0004
No transmitter	N-18	−	16	<0.0004
Uncloned neuroblastoma		−	16	0.0014
Glioma	C-6	−	15	<0.0002
L-cells	B82	−	34	<0.0001

The cloning efficiencies of the following cell lines were determined in F-14 with and without tyrosine plus 5% dialyzed fetal-calf serum: neuroblastoma clone N-115 with high tyrosine hydroxylase activity; clone NS-20, with choline acetyltransferase but no tyrosine hydroxylase activity; and clone N-18 with neither enzyme; as well as an uncloned line derived from neuroblastoma C1300, glioma clone C6, and L-cell clone B82. In most cases 100-mm petri dishes were inoculated in quintuplicate with 10^6, 10^5, 10^4, 10^3, and 10^2 cells. Colonies were scored after approximately 30 days of incubation. Duplicate experiments were performed.

TABLE 2. *Tyrosine hydroxylase activity of neuroblastoma lines selected without tyrosine*

Cell lines	pmol of $^3H^+$ released from [^{3}H]tyrosine/min per mg of protein
N-TD6	324
N-T4	162
N-T7	101
N-TN10	74
N-T13	65
N-TN1	63
N-T1	42
N-T12	34
N-TD4	33
N-T6	30
N-T16, N-TD2, N-T8, N-T14, N-T15, N-T16, N-TN2, N-TN12, N-TN16, N-TD7, N-TD10	<10

An uncloned line of neuroblastoma C-1300 with negligible tyrosine hydroxylase activity (<10 pmoles of $^3H^+$ released from [^{3}H]tyrosine per min/mg of protein) was grown in F-14 medium without tyrosine for 30 days (see Table 1). Well-isolated colonies were picked, grown, and assayed for tyrosine hydroxylase as described in *Methods*.

Tyrosine hydroxylase reactions were supplemented with 0.25 mM *N*-(3-hydroxybenzyl)-*N*-methylhydrazine, an inhibitor of aromatic amino-acid decarboxylase. Reaction products were eluted from alumina oxide columns and characterized by thin-layer and paper chromatography with four solvent systems (Table 3). The major reaction product was identical in chromatographic mobility to authentic dopa.

TABLE 3. *Chromatographic characterization of the ^{3}H-labeled product of the tyrosine hydroxylase reaction*

Cell lines	Solvent systems A	B	C	D
	% of the ^{3}H-labeled product with the mobility of dopa			
N-TD6	95	89	95	89
N-T4	96	85	97	85
N-T7	95	79	94	91
N-T1	94	87	95	89
N-115	99	95	95	93

Tyrosine hydroxylase reactions were performed as described in *Methods*. Tritiated catechol products were characterized by thin-layer chromatography with the following solvent systems: *solvent A*, 1-butanol–concentrated acetic acid–water (12:3:5); *solvent B*, methylethylketone–concentrated formic acid–water (24:1:6); *solvent C*, ethylacetate–concentrated acetic acid–water (15:15:10); *solvent D*, 1-butanol-1 N acetic acid–absolute ethanol (35:10:10). Solvent A was used with Whatman No. 1 filter paper; solvents B, C, and D were used with thin-layer cellulose plates (Eastman 6064). The R_F values of authentic dopa were similar to those reported previously. One hundred percent corresponds to the following dpm recovered after chromatography: 6060, N-TD6; 7860, N-T4; 7550, N-T7; 9590, N-T1; and 17,200, N-115. The numbers in the table correspond to the percent of tritiated product recovered with the chromatographic mobility of authentic dopa.

In addition, several unidentified radioactive products were found, which represented <10% of the tritiated reaction products. In other experiments performed with Tom Lloyd, 3-iodotyrosine and antiserum prepared against purified bovine adrenal medulla tyrosine hydroxylase (14) were found to inhibit the tyrosine hydroxylase activity of N-TD6 homogenates in a dose-dependent manner. These results, together with studies reported previously (5), strongly suggest that dopa synthesis is catalyzed by tyrosine hydroxylase.

Some, but not all, of the cell lines obtained in the absence of tyrosine showed catecholamine histofluorescence induced by formaldehyde, suggesting that catecholamines are both synthesized and stored (David Jacobowitz, paper in preparation). A small proportion of cells from the C1300 neuroblastoma tumor grown *in vivo* (15) and *in vitro* (16, 17) have been shown to exhibit catecholamine fluorescence.

DISCUSSION

The results show that neuroblastoma cell lines differentiated with respect to adrenergic neurotransmitter synthesis can be selected on the basis of their ability to grow in medium lacking tyrosine. Clones derived from various cell types, including glia and fibroblasts, were tested, but multiplication in the absence of tyrosine was found only in neuroblastoma cells with tyrosine hydroxylase activity. When uncloned populations of neuroblastoma cells were selected by this procedure, approximately one out of 7×10^4 cells gave rise to a cell line that multiplied well without tyrosine. Approximately 50% of the cell lines thus obtained had tyrosine hydroxylase activity.

Shiman, Akino, and Kaufman have shown that tyrosine hydroxylase preparations catalyze the synthesis of tyrosine and its conversion to dopa at similar rates in the presence of tetrahydrobiopterin, the *in vivo* cofactor (3). Studies of brain metabolism *in vivo* (19, 20) and in synaptosome preparations (21) suggest that phenylalanine is converted to tyrosine in the normal nervous system. The ability of tyrosine hydroxylase to catalyze tyrosine synthesis may be advantageous since adrenergic neurons thus can generate the substrate needed for transmitter synthesis by an independent endogenous route. The observation that adrenergic neuroblastoma cells support the growth of neighboring cells in the absence of tyrosine suggests that tyrosine is released by neuroblastoma cells and has a tropic effect upon L cells which determines the relative positions of these two cell types. The interaction of certain cells in the normal nervous system may be determined in a similar manner.

It should be possible to select dividing, as well as nondividing, cells on the basis of their ability to multiply or survive in the absence of tyrosine. In addition, the selection procedure coupled with somatic cell hybridization affords a means of generating and selecting different neural phenotypes on the basis of their specificity in encoding neural information. Ultimately, it may be possible to obtain five classes of cells with respect to putative neurotransmitters, those that synthesize dopamine, norepinephrine, epinephrine, serotonin, or melatonin, since tryptophan hydroxylase also catalyzes the formation of tyrosine from phenylalanine (21). E. Heldman in our laboratory has shown that one of the cell lines, N-T16, that grows in the absence of tyrosine and does not have tyrosine hydroxylase activity, does synthesize serotonin (unpublished observations).

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