*ESTABLISHMENT OF FUNCTIONAL CLONAL LINES OF NEURONS FROM MOUSE NEUROBLASTOMA**

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Abstract.—Clonal lines of neurons were obtained in culture from a mouse neuroblastoma. The neuroblastoma cells were adapted to culture growth by the animal-culture alternate passage technique and cloned after single-cell plating. The clonal lines retained the ability to form tumors when injected back into mice. A striking morphological change was observed in the cells adapted to culture growth; they appeared as mature neurons, while the cells of the tumor appeared as immature neuroblasts.

Acetylcholinesterase and the enzymes for the synthesis of neurotransmitters, cholineacetylase and tyrosine hydroxylase were assayed in the tumor and compared with brain levels; tyrosine hydroxylase was found to be particularly high, as described previously in human neuroblastomas. The three enzymes were found in the clonal cultures at levels comparable to those found in the tumors. Similarly, there were no remarkable differences between the three clones examined.

The difficulty in separating glial cells and neurons has proved to be a major obstacle in the biochemical characterization of the components of the nervous system. Methods of separation, which yield homogeneous populations of cells, are limited by the low amount of cells obtainable.[1, 2] On the other hand, the methods described for large scale preparation[3–5] give highly heterogeneous fractions and produce a large amount of cell damage.[6] Clonal cell lines of the components of nervous tissue would, therefore, provide a useful tool for the study of neurobiology. Recently, a glial cell line which retains in culture the ability to synthesize the brain specific protein S-100 has been developed from a rat brain tumor.[7] This cell line was obtained using the technique of alternate animal-culture passage described by Buonassisi *et al.*[8] to obtain in culture functional cell lines from functional tumors.

Previous work on human neuroblastomas indicates that this is a functional tumor and can adapt to growth in culture. Short-term cultures of human neuroblastoma explants have been reported to metabolize norepinephrine as "*in vivo*";[9] also long-term cultures of human neuroblastoma have been described to be able to rapidly metabolize norepinephrine.[10] Fast degradation of norepinephrine[11–12] and high tyrosine hydroxylase content[13] have been reported as biochemical features of neuroblastoma.

The availability of a transplantable mouse neuroblastoma offered the opportunity to apply the alternate animal-culture passages technique to select clonal lines which are more adaptable to the culture conditions and at the same time maintain their function. Choline acetylase, acetylcholinesterase, and tyrosine

hydroxylase activities were measured as a test of the functional capacity of the derived cell cultures.

Materials and Methods.—The mouse neuroblastoma, C 1300, was obtained from Jackson Laboratory, Bar Harbor, Maine. C-14 acetyl CoA, spec. act. 60 mc/mM, was obtained from New England Nuclear Corp., Boston, Mass.; 3,5 H-3 L-tyrosine, 36 c/mM, from Amersham/Searle Corp., Des Plaines, Ill.; Triton X-100, L-tyrosine and butyrylthiocholine from Mann Research Laboratories, New York, N.Y.; acetyl CoA, choline iodide, acetylthiocholine iodide, neostigmine methyl sulfate, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), 6-7-dimethyl-5,6,7,8-tetrahydropterine hydrochloride from Calbiochem, Los Angeles, Calif.; Dowex 50 X8, 100–200 mesh, and Dowex 1 X10, 200–400 mesh from Baker, Phillipsburg, N.J.

Tissue culture methods: The technique of alternate passage animal-culture[8] was followed to adapt the neuroblastoma cells to growth in monolayer culture. The tumor tissue was dissociated by viokase treatment and the single cells plated in Falcon plastic plates or flasks pretreated with 5% gelatin solution. The medium used was Ham's F 10[14] supplemented with 15% horse serum and 2.5% fetal calf serum.

Clonal lines were isolated following the single-cell plating technique described by Puck *et al.*[15] from cultures at the second or third passage *in vitro*. Tumors grown from clonal cultures injected into host animal will be referred to as clonal tumors and designated with the lettering of the clone of origin.

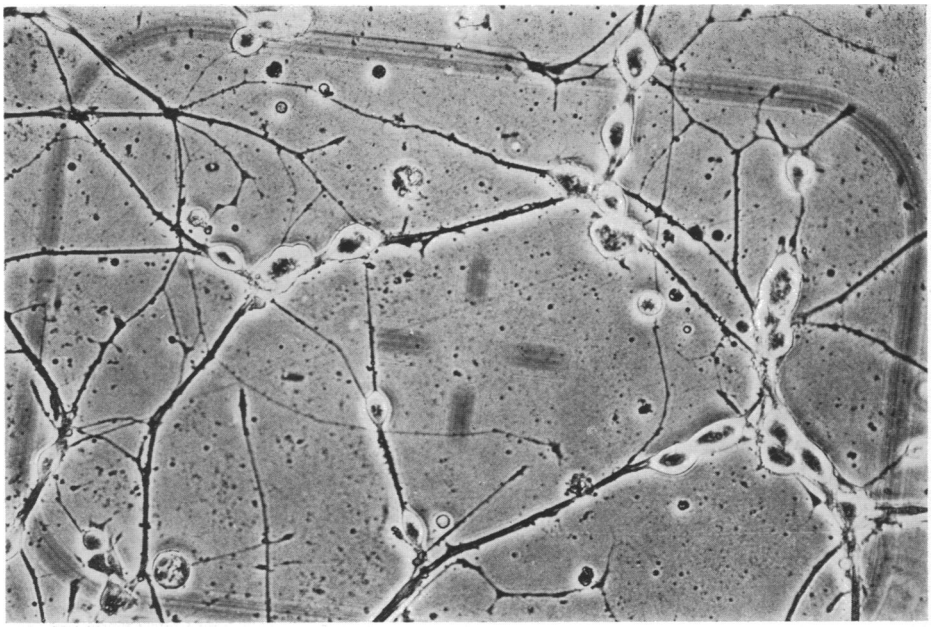
Enzyme assays: Tyrosine hydroxylase and choline acetylase were assayed as described by Wilson *et al.*[16] Acetylcholinesterase was assayed according to the method of Ellman,[17] with 1 ml as final volume of the reaction mixture. The assays were run using as substrate both acetylthiocholine (0.5×10^{-3} *M*) and butyrylthiocholine (1×10^{-3} *M*) to ascertain that true acetylcholinesterase activity was measured. Cholinesterases nonspecific for acetylcholine hydrolyze butyrylcholine at a faster rate than acetylcholine.[18, 19] The tumors were finely minced with scissors and then homogenized in the appropriate buffer in a glass homogenizer with a motor-driven Teflon pestle. Cultures were washed twice with phosphate buffered saline solution (PBS: NaCl 8 gm, KCl 0.2 gm, Na_2HPO_4 1.15 gm, KH_2PO_4 0.2 gm, $MgCl_2$ 0.1 gm, $CaCl_2$ 0.1 gm per liter at pH 7) and scraped with a rubber policeman directly in the required volume of buffer for homogenization. Alternatively, the cells were scraped in 2–3 ml of PBS, centrifuged down at low speed, and then homogenized in the required volume of buffer; this procedure was followed when several flasks were needed for an assay.

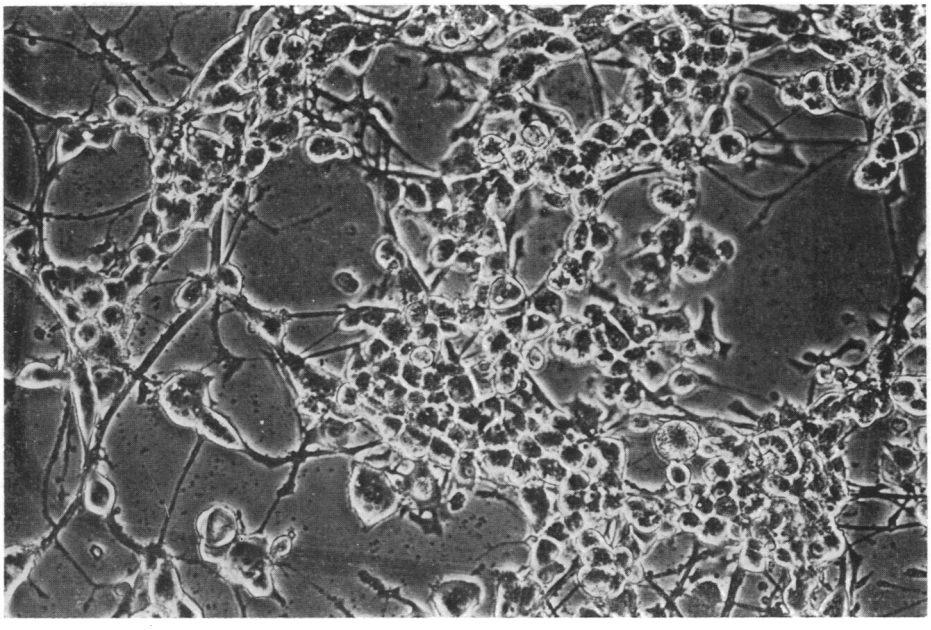
Proteins were determined by the Lowry method,[20] and all enzyme activities expressed as μmole or mμmoles of substrate converted in 10 min per milligram protein.

Results.—Morphology: Neuroblastomas have been described as highly undifferentiated tumors. The C 1300 Jackson tumor, described as a spontaneous tumor of the region of spinal cord, showed the usual morphology of neuroblastomas. Histological section of the tumor revealed the presence of only round cells. Fibers were absent.

When placed into culture, the cells undergo a striking change in morphology. The most striking characteristic of these cells is the large number of elongated processes which emanate from the cell body. These processes begin development soon after subculture or the initiation of primary culture, and within a few days form a complex network. A typical colony of a clonal line is shown in Figure 1. In each colony the cells remain rather sparse. After a few days in culture, round cells appear on the colonies (Fig. 2). They pile up on the colonies and form clumps, which tend to float away, while the cells with long processes remain attached to the plates.

Enzyme assay: The clonal line NB42B and the cultures obtained from the





FIGS. 1–2.—Phase contrast photomicrograph (×370). 4-month-old continuous culture of clone NB42B.

clonal tumor NB41A and NB41B were assayed for acetylcholinesterase, choline acetylase, and tyrosine hydroxylase activities. These results are reported in Table 1. The three enzymes were present in the original tumor. As compared

with the brain, the tumor showed a higher content of tyrosine hydroxylase and a lower content of choline acetylase and acetylcholinesterase. The three clones studied, both as tumors and in culture, did not show striking differences in their enzyme contents. The differences observed between tumor and brain were much greater. Some variations, however, seem to occur going from tumor to culture. Choline acetylase activity is lower and possibly tyrosine hydroxylase activity is higher in culture than in the tumor (NB41A and NB41B). Similarly, the clonal tumors NB41A and NB41B seem to have higher choline acetylase activity and lower tyrosine hydroxylase activity than the original tumor. Acetylcholinesterase content appears to be more constant in all the conditions.

Choline acetylase, tyrosine hydroxylase, and specific acetylcholinesterase were not detected in control culture of mouse fibroblasts (AF1—Table 1).

Discussion.—Morphological observations indicate that the neuroblastoma cells can adapt to culture conditions and, moreover, that the culture conditions stimulate the immature neuroblasts present in the tumor to complete (or at least to proceed further in) their maturation. The same observations had been described a few years ago by Goldstein,[21-22] culturing explants of human neuroblastoma.

TABLE 1. *Enzyme activities of neuroblastoma cell lines and tumors.*

	Choline Acetylase		Tyrosine Hydroxylase		Acetylcholinesterase — Acetyl		Acetylcholinesterase — Butyryl	
	Tumor	Culture	Tumor	Culture	Tumor	Culture	Tumor	Culture
Brain	8.1		0.003		1.16		0.031	
Neuroblastoma	0.206	...	0.0541	...	0.125	...	0.045	...
NB41A	0.790	0.126	0.0041	...	0.107	0.144	0.024	...
NB41B	0.840	0.275	0.0082	0.0140	0.340	0 192	0.026	...
NB42B	...	0.210	...	0.0145	...	0.247	...	n.d.
AF 1	...	n.d.	...	n.d.	...	0.034	...	0.066

Choline acetylase and tyrosine hydroxylase activities are expressed in mμmoles/10 min/mg protein; acetylcholinesterase in μmoles/10 min/mg protein; n.d. indicates that enzyme activity was not detectable at homogenate concentration at least as high as that of neuroblastoma cells. NB41A and NB41B, cultures obtained from clonal tumors of mouse neuroblastoma; NB42B, clonal line of mouse neuroblastoma; AF1, clonal line of mouse fibroblast.

The data reported in Table 1 show that the neuroblastoma cells, adapted to growth in monolayer, keep the ability of the tumor of origin to synthesize the key enzymes for the transmission of the nerve impulse. At present, it is not possible to evaluate the significance of the observed variations of enzyme activities. In fact, specific activity of enzymes in the various tumors could be affected by the variable extent of necrotic areas; on the other hand, it has not yet been determined whether the enzyme activity of the cultured cells may vary during the growth cycle and therefore, whether the measured enzyme activity is the maximal one the cells can reach under the conditions of culture. Further work is in progress to ascertain this.

Our findings of the enzymes for the synthesis of norepinephrine and acetylcholine and acetylcholinesterase in clonal cell lines is in agreement with the Burn and Rand hypothesis for the transmission of impulse in adrenergic fibers[23] and demonstrate that the two neurotransmitters are present in the same neurons.

Summary.—Clonal lines of neurons were obtained from a mouse neuroblastoma by alternate animal-culture passage technique. Choline acetylase, acetylcho-

linesterase, and tyrosine hydroxylase activities were assayed. Enzyme activities of cultures and tumors were of the same order of magnitude.

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