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Regulation of Axon Formation by Clonal Lines of a Neural Tumor

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Abstract. Clonal lines of neuroblastoma cells were found to extend or retract axons depending upon the concentration of serum. Neurite extension was not inhibited by cycloheximide but was sensitive to colchicine or vinblastine, suggesting that neurite formation is dependent upon the assembly of microtubules or neurofilaments from preformed protein subunits.

Clonal lines of neuroblastoma cells exhibit many properties of differentiated sympathetic neurons. Cells extend branched axons† up to 3000 μ in length,[1–4] possess membranes that are electrically excitable,[4] respond to acetylcholine,[5] and also contain enzymes for the synthesis and metabolism of catecholamines[1, 2, 6] and for the hydrolysis of acetylcholine.[1, 7] Cultures usually contain two types of cells quite different in morphology: round cells without processes and cells with axon-like processes. Although cell shape *in vitro* is notoriously variable and many varieties of cells are capable of extending processes, the distinctive neuronal appearance of neuroblastoma processes agrees well with the neural characteristics demonstrated in biochemical and neurophysiological studies.

The proportion of neuroblastoma cells with axons was found to vary greatly, depending upon the clone studied and the conditions of cell culture. The system is relatively simple from an experimental point of view and may be useful for studies of both the differentiation and the function of neurons.

Materials and Methods. Neuroblastoma C-1300 was adapted for growth *in vitro* as previously described.[4] Cells were first cloned as a colony from agar and were then cloned from single cells with the use of stainless steel cylinders. Cultures of neuroblastoma clone N-18 were grown in Falcon flasks or Petri dishes in Dulbecco's modification of Eagle's medium (DMEM) plus 10% fetal calf serum (except where indicated) at 37°C in an atmosphere of 10% CO_2 and 90% air.

Cells were evaluated for the presence of axon-like processes from photomicrographs (data of Figs. 4 and 5) or directly under the microscope. An initial cell concentration of approximately 3000–5000/cm^2 proved to be convenient for counting cells and axons and was used except where indicated; 300–1200 cells were counted for each value. Reproducibility was approximately ±15%.

Trypsinized cells (500–1000) were counted with a hemocytometer and viability was estimated by exclusion of nigrosin.

^{3}H-Proline and ^{3}H-thymidine incorporations into protein and DNA, respectively, were determined by addition of labeled and unlabeled compounds in Dulbecco's modification of Eagle's medium to plates to achieve the following concentrations: L-proline 10^{-3} M, 10 μCi/ml, 2850 cpm/nmole; thymidine 3×10^{-6} M, 5 μCi/ml, 600 cpm/pmole. Cells

were incubated for 1 hr at 37°C in a 10% CO_2–90% air atmosphere. Protein synthesis was determined by counting hot trichloroacetic acid-precipitable material on nitrocellulose filters. DNA synthesis was assayed by counting cold trichloroacetic acid-precipitable material on glass fiber filters (Millipore Co.). ^{3}H-Thymidine (10 Ci/mmole) and ^{3}H-proline (26.3 Ci/mmole) were obtained from Schwarz and were purified by paper chromatography prior to use. Bovine plasma protein fractions were obtained from Pentex (α_1-globulins, Cohn fraction IV_1; α_4-globulins, fraction IV_4; β-globulins, fraction III; γ-globulins, fraction II; transferrin, 67% estimated purity; bovine serum albumin, crystalline).

Results. We thought it likely that the proportion of neuroblastoma cells with axons and the length of axons might be related to the rate of cell division because cells are known to retract processes prior to cell division. Since serum is required for multiplication of neuroblastoma cells, the extent of cell division was restricted by incubating cells with relatively low concentrations of serum. Few cells possess processes in 10% fetal calf serum (Fig. 1*A*). Most cells are round and adhere to one another, forming clusters. When cells are incubated without serum, axon-like processes are rapidly extended. Within 30 to 60 min, most cells possess processes 25–100 μ in length (Fig. 1*B*); relatively long neurites are found after one day (Fig. 1*C*). After four days (Fig. 1*D*) many cells possess axons up to 2000 μ in length, and further elongation and arborization are apparent at seven days (Fig. 1*E*).

In most cases, two to four branched neurites extend from the body of a single cell. Usually a binary pattern of neurite branching was found (i.e., two neurites arise from each branch node).

The relation between serum concentration and the proportion of cells with neurites is shown in Figure 2. Approximately 1–5% of the cells extend axons in 10% serum. Most of the cells extend neurites in the presence of 0 to 1% serum; however, the rate of appearance of neurites is inversely related to serum concentration.

The effect of cycloheximide upon axon formation was studied to determine whether axon outgrowth and migration are dependent upon protein synthesis (Fig. 3). In addition, cells were incubated with colchicine or vinblastine to investigate the possibility that axon outgrowth is dependent upon the assembly of microtubule protein. Both alkaloids interact with microtubule protein.[8, 9] Cycloheximide was found to have little effect on neurite outgrowth at concentrations up to 1.8×10^{-4} *M*. This concentration of cycloheximide inhibits the incorporation of proline into protein by more than 97%. However, vinblastine and colchicine inhibit neurite out-growth completely at 10^{-7} and 10^{-6} *M*, respectively. These results suggest that neurite formation does not require *de novo* protein synthesis but is dependent upon the assembly of microtubules from performed protein subunits. In addition, electron microscopic observations demonstrate numerous microtubules, 240 Å in diameter, and neurofilaments, 100 Å in axons induced by growth in low serum.

Additional factors influencing neurite outgrowth are shown in Table 1. Neurites are not extended by cells at 3° and outgrowth is greatly retarded at 24° compared to 37°. The temperature of incubation thus markedly affects neurite outgrowth. As shown in Experiment 2, the serum factor(s) affecting neurite outgrowth is not dialyzable and is active after incubation at 100° for

15 min. Conditioned media (final serum concentration, 1–2%) also inhibits neurite formation. Cells incubated in Dulbecco's phosphate-buffered saline solution plus glucose extend processes as well as those incubated in growth medium (Expt. 3). No processes are extended in the absence of Ca^{++} and Mg^{++}.

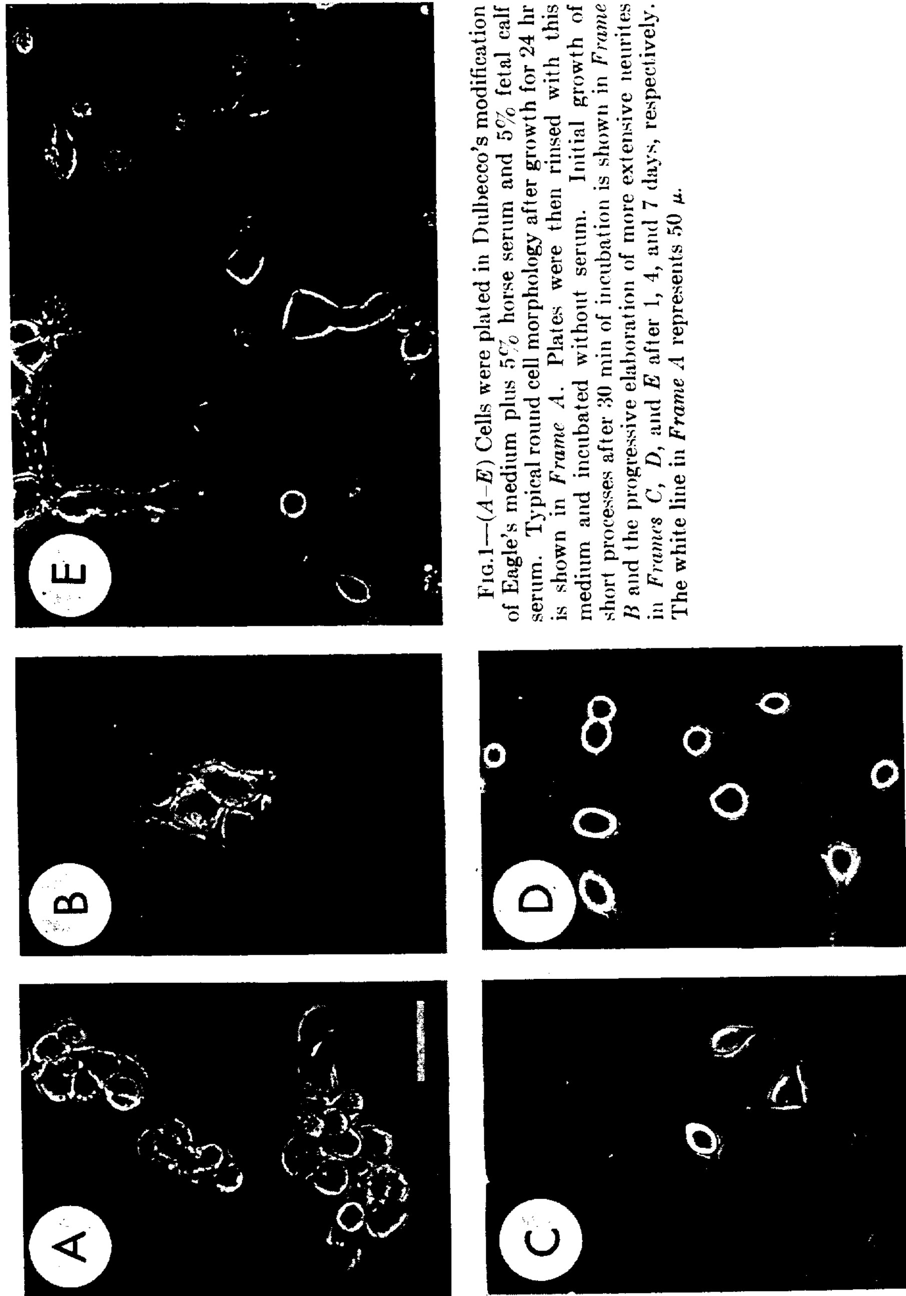

FIG. 1—(*A–E*) Cells were plated in Dulbecco's modification of Eagle's medium plus 5% horse serum and 5% fetal calf serum. Typical round cell morphology after growth for 24 hr is shown in *Frame A*. Plates were then rinsed with this medium and incubated without serum. Initial growth of short processes after 30 min of incubation is shown in *Frame B* and the progressive elaboration of more extensive neurites in *Frames C, D,* and *E* after 1, 4, and 7 days, respectively. The white line in *Frame A* represents 50 μ.

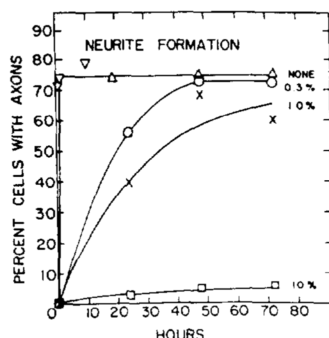

Fig. 2.—Effect of serum concentration upon rate of neurite formation. Cells were grown in Dulbecco's modification of Eagle's medium containing the indicated total concentration of an equal mixture of horse serum and fetal calf serum, and neurites were evaluated at indicated times.

△, No serum; ○, 0.3% serum; X, 1.0% serum; □, 10% serum; ▽, data obtained at 1, 2, and 8 hr in a separate experiment where serum was removed from cells previously grown in 10% serum for 24 hr.

The effects of serum protein fractions upon neurite formation are shown in Table 2. Crystalline bovine serum albumin, transferrin, and a bovine γ-globulin fraction have little effect upon the formation of neurites. However, α_1-, α_4-, and β-globulin fractions from bovine serum are inhibitory. Partial inhibition is also observed at concentrations 10 to 100-fold lower than those shown. Chondroitin sulfate, α-lactalbumin, and β-lactoglobulin also are without effect (data not shown).

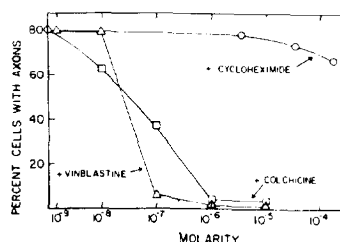

Fig. 3.—Effect of colchicine, vinblastine, and cycloheximide on initial neurite formation. Cells were grown with 5% horse serum and 5% fetal calf serum. After 24 hr plates were rinsed with Dulbecco's modification of Eagle's medium and then incubated for 2 hr in this medium and the components indicated.

An attempt was made to examine the relation between neurite extension and cell division (Figs. 4*A* and *B*). During logarithmic growth in 10% serum, less than 3% of the cells possess neurites. After incubation for two days, the culture media of some plates was replaced with fresh media with 0.1% serum. Eighty percent of the cells extended neurites during the next 24 hours. Further incubation led to a marked increase in neurite length;

Table 1. *Requirements for initial neurite formation.*

Expt. no.	Conditions	Percentage of cells with axons
1	Minus serum, 3°C	0.3
	Minus serum, 24°C	9
	Minus serum, 37°C	73
	10% serum, 37°C	1
2	Minus serum	70
	1% serum	7
	1% serum, heat for 15 min at 100°C	15
	1% serum, dialyzed	12
3	Minus serum	76
	Phosphate-buffered saline (minus serum and growth medium)	76
	Phosphate-buffered saline without Ca^{++} and Mg^{++} (minus serum and growth medium)	8

Cells were incubated 1 day in Dulbecco's modification of Eagle's medium plus 10% fetal calf serum. Plates then were washed and fresh medium plus components listed above were added. Cultures then were incubated for 2 hr at 37°.

TABLE 2. *Effect of serum fractions on initial neurite formation.*

Additions	Percentage of cells with axons
None	83
10% fetal calf serum	2
Albumin, crystalline	74
Transferrin	84
α_1-Globulin (IV-1)	9
α_4-Globulin (IV-4)	4
β-Globulin (III)	21
γ-Globulin (II)	68

Cells, incubated for 1 day in the presence of 10% fetal calf serum, were washed with Dulbecco's modification of Eagle's medium and then were incubated for 2 hr with this medium plus the components indicated (serum fractions were tested at 1 mg protein/ml of media, final concentration.)

however, the proportion of cells with neurites remained constant. On the fifth and seventh day, some cultures were stepped up from 0.1 to 10% serum; fewer cells with neurites were found after incubation. Additional results obtained by time-lapse cinematography show that neurites that detach from the surface of the Petri dish are resorbed. However, many cells retain long, well-developed neurites. Since cells with relatively long neurites are uncommon during logarithmic growth, it seems probable that some neurites are not retracted under these conditions.

The number of viable cells per plate is shown in Figure 4*B*. After a short lag, cells in 10% serum grew logarithmically; the population generation time was 16 hr. The rate of cell multiplication decreased markedly when cells were shifted down from 10 to 0.1% serum. In 0.1% serum the number of cells per plate did not increase after two days of incubation. While the addition of 10% serum on the fifth day resulted in disappearance of neurites and cell multiplication,

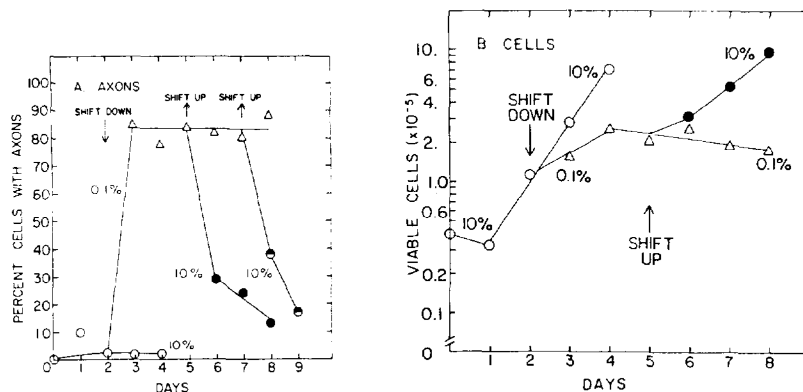

FIG. 4.—(*A* and *B*) Effect of serum on axon formation and cell multiplication. On successive days the number of viable and total cells were counted and photomicrographs were obtained for axon counts as described under *Materials and Methods*. Symbols represent the following: ○, 10% fetal calf serum; △, on the second day the cultures indicated in the figure were rinsed with Dulbecco's modification of Eagle's medium and then incubated in fresh medium plus 0.1% fetal calf serum; ●, on the fifth day fetal calf serum was added where indicated (10%, final concentration); ◓, on the seventh day fetal calf serum was added (10%, final concentration) to cultures as indicated.

the population generation times were 45 and 29 hours for the first and second generations, compared with 16 hr found with cells in logarithmic growth. Since the generation time of individual cells during logarithmic growth was also found to be 16–18 hours (by time-lapse cinematography), division by 35% of the cells could account for the prolonged population generation time. Morphological observations of such cultures support the possibility of a population of non-dividing cells with long axons. Alternatively, all cells may be capable of dividing with a prolonged generation time.

The rates of DNA and protein synthesis were also determined in the above experiment (Table 3). In general, the rate of thymidine incorporation was

TABLE 3. *Effect of serum on ^{3}H-thymidine and ^{3}H-proline incorporation.*

Growth condition	Percentage of serum	Day*	cpm (× 10^{-3}) Incorporated per 10^5 Viable Cells ^{3}H-thymidine	^{3}H-proline
Logarithmic	10	1–4	12.5 ± 1.6	3.5 ± 0.8
Step down	0.1	3–5	8.3 ± 1.1	4.3 ± 0.5
Step down	0.1	6–8	2.2 ± 0.2	1.9 ± 0.1
Step up	10	6	19.6	3.7
Step up	10	8	13.0	4.2

*Day shown in Figs. 4*A* and *B*.
Experimental conditions are given in the legend to Fig. 4. ^{3}H-Thymidine incorporation into nucleic acid and ^{3}H-proline incorporation into protein were determined as described under *Materials and Methods*. Average values ± the standard error of the mean are shown. Assays were performed in triplicate each day.

similar to the rate of cell multiplication; however, the initial rate of incorporation after cells were shifted up to 10% serum on the sixth day was high compared with the second day of the experiment. It is possible that cells were synchronized to some extent with respect to position within the cell cycle. The rate of proline incorporation into protein was relatively less affected by changes in serum concentration, being approximately 50% after prolonged incubation in 0.1% serum.

The effect of serum upon retraction of neurites was examined in a separate experiment (Fig. 5). Cells were incubated for one day without serum; approximately 84% of the cell population then possessed short processes. The addition of serum resulted in a rapid decrease in the percentage of cells with neurites that was dependent upon serum concentration. Colchicine and vinblastine also induced neurite retraction (data not shown).

The effect of serum on process formation by other cell lines was also investigated. In the presence of 10% serum, mouse L cells and HeLa cells flatten, spread, and form confluent monolayers, in contrast to the behavior of the neuroblastoma cells. L cells possessed processes in the

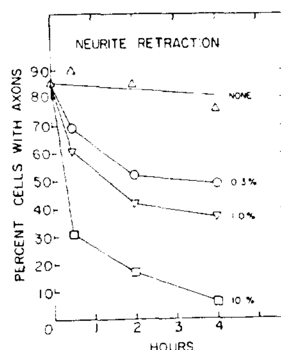

FIG. 5.—Effect of serum on neurite retraction. Cells were incubated 1 day in the presence of 10% fetal calf serum and then were incubated for another day without serum; then serum was added as indicated. Symbols represent the following concentrations of serum: □, 10%; ▽, 1%; ○, 0.3%; △, no serum.

presence of 10% serum. Removal of serum from cultures of L or HeLa cells had no discernible effect upon processes. Colchicine (10^{-6} M) inhibited the spreading of L or HeLa cells and the formation of processes by L cells. Thus L cells and HeLa cells differ from N-18 neuroblastoma cells in response to shifts in serum concentration.

Discussion. Separate clones derived from the same tumor differ markedly in the ability to extend neurites. For example, one neuroblastoma clone extends processes infrequently, whereas the majority of cells from another clone extend processes soon after they are plated. Neuroblastoma clone N-18 is particularly interesting because the proportion of cells with neurites was found to vary more than 100-fold depending upon environmental conditions.

While Klebe and Ruddle[15] have selected for populations of neuroblastoma cells with processes by elimination of dividing cells with fluorodeoxyuridine, we have devised a simple method for converting entire populations of round cells to cells with neurites that depends upon alteration of serum concentration. Serum probably affects process formation in several ways, for factors are present that influence the attachment of cells to plate, thus altering the balance between process extension and retraction, in addition to stimulating cell division, nucleic acid, and protein synthesis.[10-13] As Weiss[14] has emphasized, neurites migrate only on solid surfaces, thus the stability of interactions between cell and substratum is of great importance. The morphology of neuroblastoma cells is apparently derived by a process of selection. Neurites may explore an area 10,000 times that occupied by the cell body and neurites forming the most stable *set* of attachments relative to perturbing forces are selected. Results obtained by time-lapse cinematography show that the rate of neurite migration from neuroblastoma cells is approximately 75–125 μ/hr. However, migration is discontinuous.

Removal of serum from cultures results in axon outgrowth which may be related, at least in part, to a more stable interaction between cells and plate. However, the restriction on the rate of cell division imposed by the absence of serum probably permits the uninterrupted synthesis of relatively long neurites, since neuroblastoma cells retract processes prior to mitosis.

The relation between cell division and axon or dendrite extension may be of fundamental importance. Since 70–85% of neuroblastoma cells extend processes within 60 min after removal of serum, it is clear that cells are capable of extending processes during most of the cell cycle.

One may hypothesize that axons and dendrites migrate from most normal neurons of the central nervous system when the neurons are in the G-1 period of the cell cycle and are repressed with respect to cell division, since most neurons are diploid and do not divide. However, neurites may migrate from certain neurons, such as Purkinje cells, during the G-2 period, since these neurons are tetraploid. At least two modes of repressing neuron multiplication can thus be envisioned.

Cycloheximide does not inhibit the initial outgrowth of neurons; thus, neurite synthesis is not dependent upon protein synthesis. Colchicine or vinblastine, which bind to microtubule protein,[8, 9] completely inhibit neurite formation,

implying that neurite synthesis is dependent upon the assembly of microtubules or neurofilaments from preformed protein subunits. The formation of relatively long neurites probably is dependent upon the synthesis of additional microtubule protein subunits. These results are in accord with observations that pertain to flagellar regeneration.[16]

Olmsted *et al.*[3] have shown that mouse neuroblastoma C-1300 contains microtubule protein in relatively high concentration and that neurites are birefringent. In addition to our observations, Schubert *et al.*[2] previously demonstrated the presence of microtubules and neurofilaments in neuroblastoma cells by electron microscopy. It should be noted that cell mitosis is also dependent upon the assembly of microtubule subunits and is inhibited by colchicine and vinblastine. Since the termination of neuroblast multiplication either precedes or coincides with the initiation of axon formation, one wonders whether the sequence of events may relate, at least in some cases, to a mutual requirement for microtubule subunit assembly.

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† Axons and neurites are used synonymously to designate any cellular extension greater than 25 μ in length.

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