A Simple Method for the Growth of Mouse Neuron Monolayer Cultures[1] (36944)

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We described (1) the occurrence of an age dependent "auto-immune" CNS disease when C58/wm mice were immunized with a syngeneic transplantable lymphoma designated line I_b leukemia. A virus could not be implicated in the pathogenesis of the disease and histologic studies (1) indicated that neurons underwent inflammatory destruction. To obtain direct evidence whether inflammatory cells were in fact cytotoxic to neurons, an *in vitro* method of assay was needed. The current report describes a simple method for the growth of mouse neurons in short-term monolayer culture. The technic can be used to study the effect of a variety of cytotoxic or physiologically active agents on neurons *in vitro*.

Materials and Methods. Mice. The origin of our inbred strains of BALB/wm and C58/wm mice was reported (2). AKR/J and C57BL/6J mice were purchased from Jackson Memorial Laboratory, Bar Harbor, ME.

CNS tissues and growth media. Cord and brain tissues usually were obtained from 18 to 20 day old BALB/wm mouse embryos. Embryos were obtained from pregnant BALB/wm mice by Caeserean section, the placenta stripped away, and the brain removed from the cranium with a sterile spatula. The spinal column was dissected out and the cord expressed by gentle pressure with a spatula. Adult mice 3 wk old or more were killed with chloroform, dipped briefly in disinfectant (Osyl, National Laboratories, Toledo, OH) to remove microbial contaminants, and the brain removed aseptically. The spinal column was severed at the base of the skull (first cervical vertebra) and at the level of the lumbar vertebrae just above the pelvis. The spinal cord was expressed by injecting Hanks' balanced salt solution (HBSS) through a 20-gauge hypodermic needle inserted into the lower end of the spinal column. CNS tissues were transferred as quickly as possible to basal Eagles medium (BME) containing 0.004 *M* glutamine, 20% calf serum, 0.06% Tween 80, 600 mg% of glucose, and 50 μg/ml of Kanamycin (Bristol Laboratories, Syracuse, NY). This medium was designated selective growth medium (SGM). Tissues suspended in a minimum quantity of SGM contained in a petri dish over cracked ice were minced into 1 mm fragments and then dispersed by trituration with a 5 ml serologic pipette. Attempts to disperse minced tissues with a 1:1 solution in PBS (3) of 0.1% trypsin: 0.01% EDTA did not yield preparations of superior growth qualities. In some experiments secondary cultures of neurons were made in transfer growth medium (TGM): Ham's F10 medium (Grand Island Biological Co., Grand Island, NY) contained 10% fetal and 10% normal calf serum, 100 μg/ml streptomycin, and 100 units/ml penicillin.

Growth medium additives. The following reagents were tested to determine whether they enhanced the growth of mouse neurons *in vitro*: agamma calf serum (Microbiological Ass., Bethesda, MD); fetal calf serum, fetuin, and alpha-*l*-protein (Grand Island Biological Co., Grand Island, NY); EDTA and adenosine 3′,5′-cyclic monophosphate (cyclic AMP) from Nutritional Biochemicals Corp., Cleveland, OH; α-L-lecithin, phosphatidyl-L-serine, and cephalin (General Biochemicals, Chagrin Falls, OH); trypsin (1:250), peptone broth, tryptose phosphate broth, and yeast extract

[1] Supported by Grant NS 11894 from the National Institute of Neurologic Diseases and Stroke.

TABLE I. Effects of Reagents Added to Growth Medium (BME) Used for Neuron Cultures.

Reagent	Principal effects[a]
Tween 80, 0.06%	Appeared to be selective for neurons and markedly enhanced their morphologic properties.
Cyclic AMP,[b] 0.1 to 0.5 *M*	Increased the survival time of neurons in second or third transfer cultures but had no detectable effect on primary cultures.
NGF,[c] 0.2 to 10%; or trypsin, 500 to 2000 μg/ml	Improved the morphology of neurons, enhanced axon and dendrite development, and slightly increased cell numbers compared to controls.
Glucose, 600 mg%	Appeared to enhance neuron survival during tissue preparation and was selective for neurons in primary cultures.

[a] All experiments for each additive were repeated 3 to 6 times.
[b] Adenosine 3′,5′-cyclic monophosphate.
[c] NGF = nerve growth factor.

(Difco Laboratories, Detroit, MI); dimethyl sulfoxide (Merck, Sharp and Dohme, West Point, PA); DEAE (Pharmacia, Uppsala, Sweden); sodium dodecyl sulfate (SDS) from Fisher Scientific Co., Pittsburg, PA; recrystallized bovine pancreatic insulin, B grade (Calbiochem, Los Angeles, CA); lactalbumin hydrolysate (Sheffield Chemical Co., Norwich, NY); and inositol (Abbott Laboratories, North Chicago, IL). Embryo extract (EE) was prepared from 18 to 20 day old BALB/wm embryos by homogenizing them in HBSS (10%, w/v) for 10 min in a Ten Broeck homogenizer. The homogenate was centrifuged for 15 min at 1500 rpm (International clinical centrifuge) and the supernatant fluid designated EE. Nerve growth factor (NGF) was prepared essentially as described by Cohen (4) from the submaxillary salivary glands of 4 to 6 week old BALB/wm mice. Purification was carried out only as far as the alcohol precipitation step.

Staining technics and microscopy. Cells were grown on 9 × 22 mm glass cover slips, fixed in absolute methanol or 10% (v/v) formalin, and stained by the May–Grunwald–Giemsa technic (3). For phase microscopy cells grown on cover slips were washed once with PBS, inverted, placed on a microscope slide, and examined with the phase optics of an Ultraphot II Zeiss microscope. The trypan blue technic (3) was used to determine cell viability.

Results. Basic methodology for growth of primary cultures. The rapid processing of tissues in petri dishes over cracked ice reduced glucose catabolism and appeared to preserve neuron viability. Phase microscopy revealed the presence of many clumps of neurons in tissue suspensions but attempts to disperse them further by a variety of methods, including exposure to graded concentrations of trypsin or EDTA, produced cells with inferior growth qualities. Density gradient separation of mouse neurons from suspensions as described for rats by Johnson and Sellinger (5) yielded preparations that would not grow in primary culture. Cell suspensions made up in BME not containing 600 mg% glucose and 0.06% Tween 80 grew luxuriantly but consisted of mixed cell types. An important factor in the growth of neurons was the use of a thin film of calf serum in the 60 mm plastic tissue culture plates employed as primary culture vessels, *viz*, cell suspensions were allowed to attach for 30 to 60 min at 37° in a CO_2 incubator (5% CO_2–95% air) and then 4 ml of SGM medium was added. After 4 to 5 days of incubation (37°, CO_2 incubator) when many cells and tissue fragments had attached, the supernatant cell suspension was decanted and fresh medium added. The supernatant fluid was centrifuged at *ca.* 1500 rpm for 15 min, the pellet was resuspended in 5 ml of fresh SGM, and reseeded into 5 to 6 fresh tissue culture plates. Thus a second crop of attached cells was obtained from the tissues used for primary cultures.

A variety of reagents were added to BME to enhance neuron growth, *i.e.*, to enrich the concentration of neurons in primary cultures

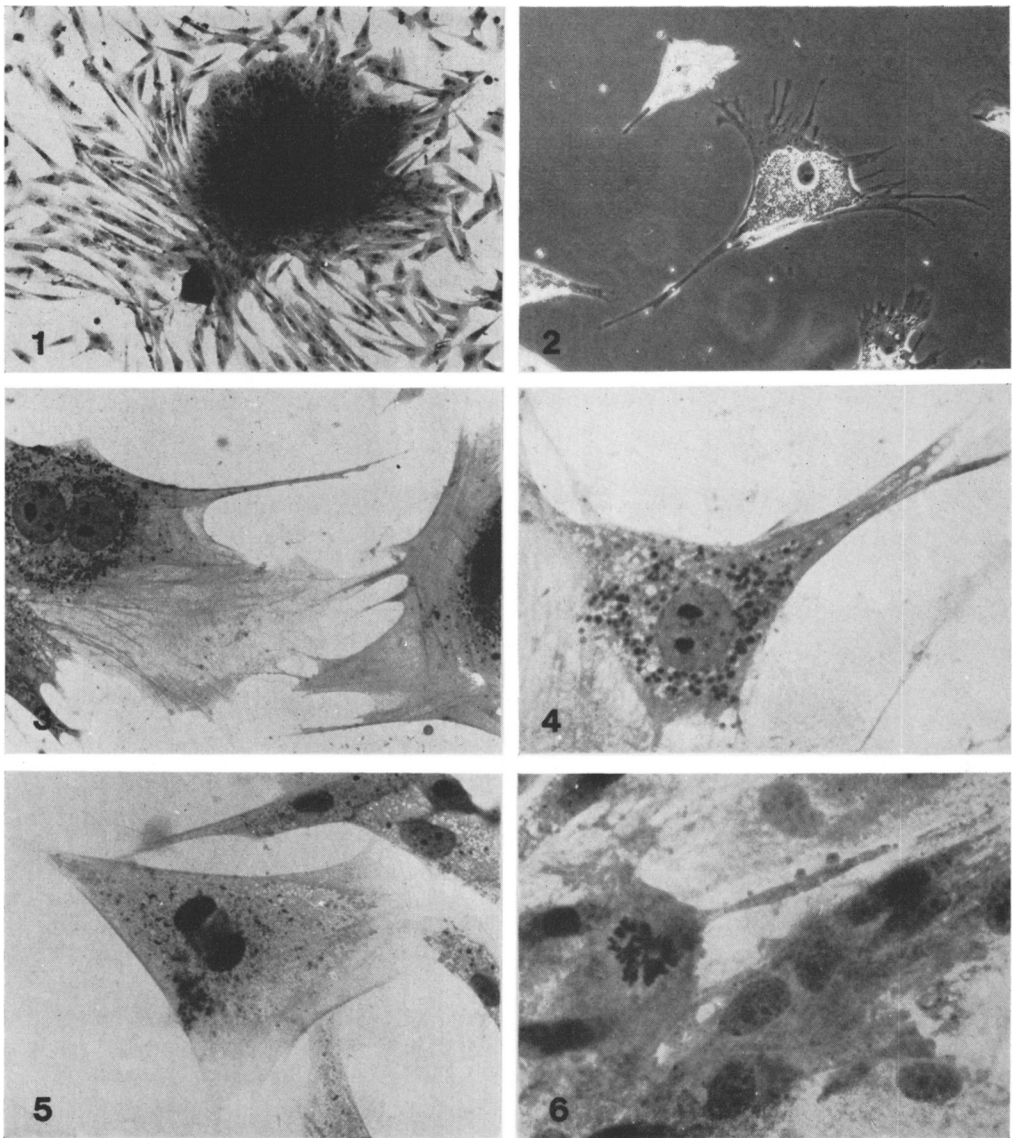

FIG. 1. Outgrowth of neurons from a fragment of BALB/wm brain tissue after 4 days in primary culture at 37° (×1050).

FIG. 2. Phase contrast photograph (×2100) of a typical neuron showing axon and dendrite-like processes (11 day old primary culture of BALB/wm spinal cord).

FIG. 3. Typical neuron from a BALB/wm spinal cord 6 days after cells were transplanted as secondary cultures (×3640).

FIG. 4. Typical neuron from BALB/wm spinal cord 6 days after secondary culture (×6440). Nissl granules and an axon-like structure are illustrated.

FIG. 5. A neuron (×3990) with a dividing nucleus (4 day old primary culture of BALB/wm spinal cord).

FIG. 6. A 4 day old secondary culture of BALB/wm neurons illustrating a nucleus in mitosis (culture was treated for 4 hr at 37° with 0.001 *M* colchicine). (×7112).

TABLE II. Growth of Neurons from Mice of Different Ages and Inbred Strains.

Inbred strains	Age[a] (mo)	Growth of neurons from: Brain	Spinal cord	Fraction of positive cultures (%)
C57BL/6J	1	0/2	2/2	
	3	1/2	1/2	
	6	1/3	1/3	8/22 = 36
	8	1/2	0/2	
	15	0/2	1/2	
Av		3/11	5/11	
BALB/wm	1	1/1	0/1	
	6	1/4	2/3	
	12	0/2	1/2	8/17 = 47
	15	1/2	2/2	
Av		3/9	5/8	
C58/wm	1	2/3	3/3	
	3	0/2	2/2	
	6	0/3	1/3	
	9	2/3	2/3	16/26 = 61
	12	2/2	2/2	
Av		6/13	10/13	
AKR/J	2	0/1	0/1	
	4	1/2	1/2	
	6	0/2	1/2	4/12 = 33
	13	0/1	1/1	
Av		1/6	3/6	

[a] For each individual experiment a control was done in parallel by use of BALB/wm embryonic mice to prove that the lot of growth medium used supported the growth of neurons.

and improve their growth, morphology, and survival time. Surface tension depressants were used (α-L-lecithin, phosphatidyl-L-serine, cephalin, Tween 80, SDS) that might disperse clumps of neurons, have a beneficial nutritional effect, and be selective against glial and other nerve tissue cell types. Known stimulators of cell attachment and growth also were tested (fetal calf serum, fetuin, insulin, cyclic AMP, NGF, *etc.*). A complete listing is given in *Materials and Methods*. Table I lists the reagents which had a clearly recognizable effect. The reagents not listed were tested over a wide range of concentrations but failed to clearly enhance the growth of neurons. Substitution of rabbit, horse, agamma calf, or fetal calf serum for ordinary calf serum did not improve results.

In the present study cells were identified as neurons (6, 7) on the basis of their morphology, *viz*, large triangular cells with a large nucleus and prominent nucleolus. Usually each cell had 1 or more thick, essentially straight, axon-like structures 30 μm or more in length. The opposite side of the cell had short dendrite-like structures. Bulbous thickenings occurred along and at the end of the axon-like process. Cells stained blue with a supravital stain (methylene blue, 1:50,000) as described by Costero and Pomerat (6). Stained cells contained numerous cytoplasmic vacuoles. Nissl granules were found in secondary cultures (10 to 15 days after cultures had been initiated) in May–Grunwald–Giemsa stained preparations. Figure 1 shows the outgrowth of neurons from a brain tissue fragment. Figure 2 shows (phase contrast) a neuron with axon and dendrite-like structures. Figure 3 illustrates the features of typical spinal cord neurons. Figure 4 shows a neuron containing many nissl granules. Figure 5 shows a neuron (not treated with colchicine) with a dividing nucleus. Figure 6 shows a typical neuron in mitosis.

Growth of neurons from mice of different ages and inbred strains. The objectives of

TABLE III. Multiplication of Mouse Neurons in Transfer Growth Medium.

Expt no.	No. of viable neurons/plate containing:[a] SGM	TGM
1	210,000	500,000
2	110,000	240,000
3	450,000	740,000
4	240,000	600,000

[a] Plates received an inoculum of 200,000 to 400,000 viable cells and were incubated at 37° for 24 hr in SGM. In half of the cultures SGM then was replaced with TGM. Cultures then were incubated for 3 days at 37° and viable cell counts done. In each experiment 2 to 4 plates were counted/assay. Results are representative of 7 experiments.

these experiments were to determine whether the age of embryos significantly affected the growth capacity of neurons and whether neurons could be grown from the spinal cord and brain of adult mice of various ages. Growth of embryonic CNS tissue was scored "good to excellent" if monolayers formed in 4 to 6 days after cultures were inoculated and if cells grew well when transplanted as secondary cultures. Cultures scored "fair" grew well enough to form diffuse monolayers but did not contain enough cells for growth as secondary cultures. "Poor" primary cultures contained only scattered cells. Mice from 22 litters, ranging in weight from 0.3 to 1.3 g/embryo, were studied. No significant effect of embryo size (age) on neuron growth was demonstrable. Embryos 18 to 20 days old thus were used as routine. The capacity of neurons to grow from the CNS tissues of mice of different inbred strains, ranging in age from 1 to 15 mo, was studied. Growth was defined to mean the presence of 1 or more colonies containing not less than 4 healthy neurons/colony. Table II shows that colonies of neurons could be grown out from mice of all strains at all ages. However, neuron growth from CNS tissues of all mice more than 2 wk of age was too sparse for transfer or practical use. The data in Table II indicate that results more often were positive with spinal cord than brain tissue. Because of the variability in results we were not convinced that neurons from C58 mice grew better than those from other inbred strains (Table II).

Secondary cultures. Four to 7 days after inoculation, primary cultures were harvested by exposure to a 1:1 mixture of 0.1% trypsin:0.01% EDTA in PBS. Residual cells were removed with a sterile rubber policeman. Cells in suspension were sedimented (1500 rpm for 15 min), resuspended to give monodisperse preparations in SGM, and seeded into 35 mm plates at a concentration of 2×10^5 cells/plate. Cells were allowed to attach for 30 to 60 min at 37° in a thin layer of calf serum contained in recipient plates, then 2 ml of SGM was added. After attachment cells sent out cytoplasmic processes and formed a diffuse monolayer in 2 to 3 days. In good cultures it was possible to show by daily cell counts that cells underwent 1 to 4 divisions. Cell viability (trypan blue dye exclusion) was *ca* 90% after incubation at 37° for 7 days. Most cultures did not survive for more than 2 to 3 serial transfers over a 3 wk period. However, some survived up to 6 serial passages over a 6 wk period. If the initial cell inoculum was $<2 \times 10^5$ neurons/plate, monolayers did not form in 2 to 3 days. If the inoculum was $<5 \times 10^4$ cells/plate, there was essentially no growth. Superior growth or/and survival was obtained in secondary cultures (Table III) if cells from primary cultures were seeded in SGM which was replaced 24 hr later with TGM.

Discussion. This report describes a simple method for the *in vitro* cultivation of neurons in monolayer culture. Our aim was to develop a method to directly study the effect of sensitized lymphocytes (1) from mice with an auto-immune CNS decrease on neurons *per se*. Thus, the report does not purport to be a detailed study of the morphologic or physiologic features of the cells themselves. The identification of the cultured cells as neurons is based primarily on the morphologic criteria used by many investigators (6–9). Typically, cultured cells were large, triangular, had a large nucleus and prominent nucleolus, and grew in a dispersed pattern. The growth pattern of cells was typical of neurons, *viz*, slow migration outward from small tissue fragments with limited multiplication in subsequent subpassages. Nissli gran-

ules were uncommon in young primary cultures (4 to 7 days after initiation) but were common in cells after the first subculture (7 to 15 days). All of the cells stained with methylene blue used as a supravital stain. The importance of this criterion in the identification of neurons was emphasized by Costero and Pomerat (6). Axon-like structures extended from one side of the cells and dendrite-like processes from the other. The axon-like structures often had bulbous thickenings and formed junctures with contiguous cells. We did not keep cultures long enough for the full development of these structures. Further evidence that the media and procedures used were selective for neurons was obtained from studies of adult mice, *viz*, when growth occurred it was restricted to the cells with the morphologic features of neurons.

Three lines of evidence are presented to indicate that neurons underwent limited multiplication *in vitro*: increase in cell number on transfer (Table III), detection of mitotic figures in cells (Fig. 6), and the occurrence of nuclei in various stages of division (Fig. 5). These results are not too surprising since neuroblasts contained in embryonic tissues could be expected to divide 2 to 4 times *in vitro*. We did not obtain evidence that neurons from adult mice multiplied *in vitro*. However, both Murray and Stout (10) and Geiger (11) reported that fully differentiated neurons were capable of dividing *in vitro*. References (6 and 7) review the rather large literature on this subject. One important limitation of the current technic was that cells were not produced in sufficient quantities for routine direct biochemical analyses. Further modifications of the culture procedure may remedy this difficulty.

Summary. A simple method is described for the preparation of primary and secondary monolayer cultures of neurons from embryonic mouse brain and cord tissues. Key points of technic were the use of culture dishes containing a thin layer of serum to facilitate cell adherence and a medium selective for neurons that contained 600 mg% of glucose and 0.06% Tween 80. Evidence is presented to show that neurons underwent limited cell division *in vitro*. Primary and secondary cultures survived 10 to 12 days. Cultures could be transferred 2 to 6 times.

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Received Sept. 6, 1972. P.S.E.B.M., 1973, Vol. 142.