Methods for Serum-Free Culture of Neuronal and Lymphoid Cells

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Preface

The growth of interest in neurobiology has been dramatic during the last decade. A significant part of this interest has been in the development of appropriate in vitro models useful for characterizing the cell-cell interactions so necessary to neuronal function, and other in vitro models which might provide systems for characterizing diffusable substances involved in neuronal differentiation and function. The problem of approaching in vitro models for these processes has been the use of the conventional serum-containing medium. As is well known, neuronal-origin tumor cell lines have been available for some time. Both human and mouse neuroblastoma lines have been established, as have adrenal medulla pheochromocytoma cells and rat glial lines. All of these lines have been shown to express neuronal-specific functions, and have at least in part provided permanent systems for study of the hormonal control of selected functions. One of the major problems encountered with neuronal systems in vitro has been that use of serum-containing medium has tended to mask the expression of the tissue-specific functions. For example, the neuroblastoma cell lines express axon extension, membrane polarization, and neurospecific enzyme activities to a greater degree in serum-free medium than in medium supplemented with this animal fluid. Also, the presence of nonphysiological serum or plasma (neuronal tissue is not normally exposed to most of the components of plasma) represents a considerable potential for altering responses without recognizing that the effective agents are not natural effectors of neuronal function.

In this volume, these problems are addressed by establishing serum-free hormonally defined media for cells of neuronal origin. Methods of culturing neuroblastoma, pheochromocytoma, and glial cells are described, as are methods for the assay and isolation of neurotrophic agents and the physiologically important trophic agent—nerve growth factor. Also included are methods for culture of neural crest-derived melanocytes and approaches for the study of the hormone responses and differentiated properties of these cells. In addition, this volume contains a chapter on the application of serum-free methods to the culture of functional differentiated mouse hypothalamic cells. Since this tissue has such a

central role in transmission of nervous system information to the pituitary, establishing methods to study regulation of hypothalamic function in vitro is an important advance. These and all of the other methods described in the first part of this volume are presented both for immediate use and, hopefully, as starting points for further development of defined media refined to suit individual needs.

The second section of this volume describes the application of serum-free defined media to culture of lymphoid origin cells. Since the growth and function of lymphoid and other hematopoietic cells has been shown to require many polypeptides, collectively named lymphokines, chapters have been included describing the preparation of three of these (T-cell growth factor [TCGF], Interleukin-2, and thymosin). Application of these factors to serum-free defined media is in a rapid state of development and it is anticipated that now that these factors are available generally, their usefulness will increase.

Of the recent applications of serum-free defined media, none has received more attention than three related to growth of normal lymphocytes, leukemia cells, and the growth of antibody-producing hybridomas in serum-free culture. Growth of normal lymphocytes is now readily studied without the conflicting agents present in serum. This has been a major advance with considerable future applications in lymphokine research and isolation. Equally, study of leukemia cells under defined conditions will facilitate the identification of phenotypic and genotypic changes from the normal lymphoid counterparts. Finally, one of the most exciting applications has been development of serum-free defined media for growth of hybridomas in vitro. As is evident from the expanding literature on the subject, the types and numbers of hybridomas being sought are enormous. One of the major problems with this technology has been the difficulty of isolating pure monoclonal antibodies from mouse ascites fluid or from the serum-containing medium of the hybridoma cell lines. With use of the serum-free defined media described in this volume, these problems can be overcome without laborious purifications.

The methods described in this book are presented in sufficient detail for immediate application to ongoing projects; they are intended as well to provide the start toward methods that can be modified to suit each investigator's needs. Detailed methods for preparation of some of the media, supplements, and substrata not directly addressed in this volume may be found in Volume 1 of this set. Methods for serum-free culture of other cell types and methods for preparation of some specialized media supplements are also discussed in Volumes 2 and 3.

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Culture Methods for Growth of Neuronal Cell Lines in Defined Media

Jane E. Bottenstein

Conventional cell culture media for continuous cell lines generally consist of a basal synthetic medium supplemented with undefined biological fluids, usually fetal calf or horse serum. When serum is present in the medium, modification of the surface of standard polystyrene tissue culture dishes is not generally required for good attachment and growth. Because of our interest in defining the specific growth requirements of neurons and in analyzing factors derived from them that influence the growth and/or differentiation of neural cells, we formulated a serum-free defined medium for a continuous neuronal cell line: the B104 rat neuroblastoma of central nervous system origin [Bottenstein and Sato, 1979, 1980]. Our approach was to replace the undefined portion of the culture medium with purified hormones, growth factors, and other substances. This chapter will describe in detail the methods for culturing neuronal cell lines in defined medium on substrata modified to permit or enhance growth.

METHODS AND MATERIALS

Water

A very important component of defined medium and other cell culture reagents is high-quality water. We recommend triple-glass-distilled water or the equivalent. We are currently using a water purification system obtained from Millipore Corp. (Bedford, MA): Milli-RO4 (prefilter and reverse osmosis) unit in series with a Milli-Q (carbon and ion-exchange cartridges) unit and final

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filtration through a 0.22 μm Twin-90 unit. Water for preparing culture media and other solutions is generated just prior to use and has a resistance of 10–18 megohm-cm. High-quality water is essential, as cells in defined medium are more vulnerable to toxic contaminants that may be bound by serum molecules.

Synthetic Basal Medium

Although liquid media may be purchased, we prepare it from the powder. Dulbecco's modified Eagle's medium and Ham's F12 medium are purchased in 1-liter packages. Appendix A shows the reagent concentrations. Powdered medium is dissolved in high-quality water at room temperature, HEPES buffering agent is added, and after mixing the pH is adjusted to 7.3 with 10 N NaOH or 10 N HCl as required. No antibiotics are added, as they are unnecessary when good cell culture techniques are used. Finally, $NaHCO_3$ is added and, after mixing again, the medium is sterile-filtered with Nalgene 0.2 μm vacuum-filtration units (#F3200-1 or #F3200-5: American Scientific Products, Houston, TX). For large volumes of medium (≥10 liters), pressure filtration by a Twin-90 unit with a dispensing bell (#PMGS 09002: Millipore Corp., Bedford, MA) is suggested. Medium is stored at 4°C in 100- to 250-ml aliquots.

Supplements

Appendix B describes the concentrated stock solutions used in making up the defined medium. Highly purified and glucagon-free insulin from Eli Lilly gives a dose-response curve identical to that of the insulin obtained from Sigma. Insulin stock solutions must be stored at 4°C only and shou'd be used within 6 weeks of preparation. Spectrographically pure sodium selenite (or selenous acid) is recommended for the best results; a reagent less pure may contain toxic contaminants. Defined medium is made up just prior to use from synthetic basal medium and concentrated supplements. We do not store defined medium, since we have noted some loss of growth-stimulating activity when it is stored at 4°C for 1 week. To minimize adsorptive losses, plastic (polypropylene or polystyrene) flasks, tubes, pipets, and micropipet tips are used when handling defined medium or supplements.

Culture Substratum

Culture vessels. Polystyrene tissue culture dishes, flasks, and roller bottles are obtained from Falcon Plastics (Cockeysville, MD), Corning (Houston, TX), or Costar (Cambridge, MA). In general, we have not found any substantial differences in these products in their ability to support growth of cells. Costar vessels are not recommended for photomicrographs, how-

ever, as lines may be apparent. Inferior growth is observed in defined medium if petri dishes rather than tissue culture dishes are used.

Modification with poly-D-lysine. Culture vessels are coated with a 0.05 mg/ml solution of poly-D-lysine (see Appendix A) for 5 min at room temperature, followed by a sterile H_2O wash, and immediate use. For example: a) 0.5 ml poly-D-lysine stock solution per 35-mm (8-cm^2) dish is followed with a 2-ml sterile H_2O wash or b) 30-ml poly-D-lysine stock solution per 490-cm^2 roller bottle is followed with a 120-ml sterile H_2O wash. Wetting of the entire surface is accomplished by tilting or shaking dishes and flasks or by putting bottles in a roller apparatus (for example, a Wheaton roller culture apparatus at setting 5). Precoated vessels may be stored for short periods of time in sterile H_2O.

Modification with fibronectin. Fibronectin is prepared from human plasma by affinity chromatography at room temperature on a gelatin-Sepharose column [Engvall and Ruoslahti, 1977; Ruoslahti et al., 1978]. Fibronectin is eluted with 4 M urea (ultrapure #821627: Schwarz/Mann, Orangeburg, NY), collected in polypropylene tubes, and assayed for protein [Sedmak and Grossberg, 1977]. Peak fractions are combined, and the concentration is adjusted to 0.25–1.00 mg/ml with 4 M urea. After dialysis overnight against 1 M urea in phosphate-buffered saline at 4°C, fibronectin is sterile-filtered and the final protein content is determined. From 250 ml of human plasma we usually obtain 10–20 mg of fibronectin. This stock solution is stored at 4°C in aliquots and it retains activity after 4 months. Purity is greater than 98% as judged by polyacrylamide gel electrophoresis. Concentrations of 1–10 μg/ml are usually optimal; some variation in activity occurs between batches. Fibronectin is added directly to culture vessels containing medium prior to the cell inoculum. Vessels are tilted immediately to ensure even distribution of the fibronectin. Similar results are obtained if dishes are precoated with fibronectin and medium is added afterward, but not if fibronectin is added after the cell inoculum [Bottenstein and Sato, 1980]. Commercial sources of fibronectin are available (Collaborative Research, Sigma, Bethesda Research Labs, and others), but we find that their activity is less than that of fibronectin prepared in the laboratory as detailed above.

Subculture and Maintenance of Cells

Cells are detached from the substratum with 0.05% trypsin/0.5 mM EDTA after washing with Hanks' balanced salt solution without Ca^{++} and Mg^{++} (see Appendix A). Excess trypsin/EDTA solution is aspirated, leaving a film on the cells. A vigorous tap after 2–4 min at 37°C is usually sufficient for cell detachment. To inactivate trypsin, detached cells are removed with a 0.05% soybean trypsin inhibitor solution (see Appendix A). A cell pellet is

obtained by centrifugation at 1,000 rpm for 2 min at room temperature, and the pellet is resuspended in culture medium.

Neuronal cell lines can be maintained in defined medium with medium changes every 5 days or sooner if the pH becomes too acidic. Between medium changes, pH may be adjusted with sterile 0.5 N NaOH or 0.5 N HCl as required. Cells may be serially subcultured by the above methods in defined medium just prior to confluence.

Cryopreservation

Continuous neuronal cell lines may also be stored in the frozen state in defined medium. Cells are detached from the substratum as above, they are resuspended in defined medium containing 10% dimethyl sulfoxide (DMSO; #D-5879: Sigma Chemical Co., St. Louis), and 1-ml aliquots are placed in freezing vials (Nunc #1076: Vanguard, Neptune, NJ). Our standard temperature step-down protocol is 4°C for 30 min, −20°C for 1.5 h, −80°C for 1 h, and then storage in the vapor phase of a liquid-nitrogen cell storage tank. Cells are thawed by gently shaking the freezing vial in a 37°C water bath, wiping the vial with an alcohol pad, resuspending the contents in defined medium, and plating on an appropriately modified substratum. After the cells have attached to the substratum (usually overnight), the DMSO-containing medium is replaced with defined medium. It is not necessary to centrifuge the thawed cells before plating to remove the DMSO-containing medium, as long as it is removed before 18 h have elapsed. The toxic effects of DMSO may vary according to the cell type or the particular cell line.

Source of Cell Lines

B104 rat neuroblastoma cells were obtained from D. Schubert (Salk Institute, La Jolla, CA); N1E-115 and NS20 mouse neuroblastoma cells from M. Nirenberg (NIH, Bethesda, MD); LA-N-1 human neuroblastoma cells from R. Seeger (University of California, Los Angeles); CHP 134 human neuroblastoma cells from H. Schlesinger (Children's Hospital, Philadelphia); PC12 cells from L. Greene (New York University, New York); and NX31 cells from W. Shain (AFRRI, Bethesda, MD).

GROWTH OF NEURONAL CELL LINES

Effect of Different Ratios of Basal Media

Synthetic basal media differ in their constituents (see review by Bottenstein [1983a]), and several of them lack important vitamins that can be supplied in adequate amounts by serum. But even in the presence of serum, differences in the growth of cells can occur in different basal media. We find that division of B104 rat neuroblastoma and LA-N-1 human neuroblastoma is

optimal in serum-containing medium if a 1:1 mixture of DME and F12 is used (Table I). Either medium alone does not give the best growth. PC12 rat pheochromocytoma cells possess many characteristics of sympathetic neurons, and these cells show a marked preference for DME medium alone, as do several glial cell lines [Michler-Stuke and Bottenstein, 1982]. These preferences are magnified in defined medium.

Growth in Defined Medium

To define a serum-free medium for growth of neuronal cell lines, we tested several representatives from the following classes of compounds for growth-stimulating activity: protein hormones, steroid hormones, prostaglandins, thyroid hormones, growth factors, polyamines, neurotransmitters, neuropeptides, trace elements, vitamins, fatty acids, and depolarizing agents. We find that only five supplements are required to support the growth of all neuronal cell lines tested thus far: insulin, transferrin, progesterone, putrescine, and sodium selenite. Three combinations of these supplements are shown in Table II: N1, N2, and N3. They differ only in the concentration of transferrin.

Defined supplements are added to the basal medium determined to be optimal for the particular cell line (not determined for NX31), and Table III shows the supplement mixture and basal medium that supports proliferation

TABLE I. Growth of Neuronal Cell Lines in Different Ratios of DME and F12 Medium

	Neuronal cell line	
DME:F12	B104 cells/dish (%)	LA-N-1 cells/dish (%)
1:0	100 ± 3	100 ± 8
3:1	ND	93 ± 10
1:1	147 ± 1	109 ± 15
1:3	ND	86 ± 9
0:1	104 ± 1	82 ± 10

Cells were grown in serum-containing medium: 10% fetal calf serum for B104 rat neuroblastoma cells and 30% fetal calf serum for LA-N-1 human neuroblastoma cells. Cell inoculum: 50,000 B104 cells per 60-mm dish and 200,000 LA-N-1 cells per 35-mm dish. Trypsin-detached cells were enumerated with a Coulter Counter on day 4 after plating. Values are expressed as the percentage of the mean number of cells in DME only (1:0) ± SD of triplicate cultures. Abbreviations: DME, Dulbecco's modified Eagle's medium; F12, Ham's F12 medium; ND, not determined.

TABLE II. Defined Medium Supplements for Neuronal Cells

Supplement	N1	N2	N3
Insulin	5 μg/ml	5 μg/ml	5 μg/ml
Transferrin	5 μg/ml	100 μg/ml	50 μg/ml
Progesterone	20 nM	20 nM	20 nM
Putrescine	100 μM	100 μM	100 μM
Sodium selenite	30 nM	30 nM	30 nM

Supplements are added to basal medium on various substrata (see Table III). Values given are the final concentrations in the defined medium. Supplements are added to basal medium in concentrated form (see Appendix B).

of the indicated neuronal cell line. All of the cells were plated on surfaces modified with poly-D-lysine and fibronectin. Optimal growth of B104 cells occurs if both modifications are made, whereas LA-N-1 cells grow well on poly-D-lysine surfaces and only slightly better if fibronectin is also present. Thus, substratum requirements may differ quantitatively. The stringency of supplement requirements is as follows: a) B104 cells: transferrin > insulin > sodium selenite = progesterone = putrescine; b) LA-N-1 cells: transferrin > insulin > sodium selenite > progesterone > putrescine; and c) PC12 cells: insulin > transferrin = sodium selenite > progesterone = putrescine. The requirement for sodium selenite is not usually evident until 4–5 days after plating. These defined culture conditions do not support the growth of nonneuronal cell lines [Bottenstein, 1980], suggesting their neuronal specificity. In addition, the supplement mixtures shown in Table III can successfully maintain postmitotic neurons with suppression of nonneuronal cell division (see review by Bottenstein [1983b]).

In general, the cells show enhanced morphological differentiation in defined medium, i.e., process formation. Figure 1 shows the morphology of B104, LA-N-1, CHP 134, N1E-115, and PC12 cells cultured in N2 medium. It should be noted that in N2 medium process formation in PC12 cells occurs in the absence of nerve growth factor (NGF), but in serum-containing medium NGF is required for process extension.

In attempting to define the growth requirements of neuronal cell lines, we encountered many substances that were growth-inhibiting. Table IV lists the concentration or range of concentrations that were inhibitory to B104 and/or LA-N-1 neuroblastoma cells. In vivo cells may encounter both growth-stimulating and growth-inhibiting influences, and it is the integrated response that determines the actual growth rate at a particular locus.

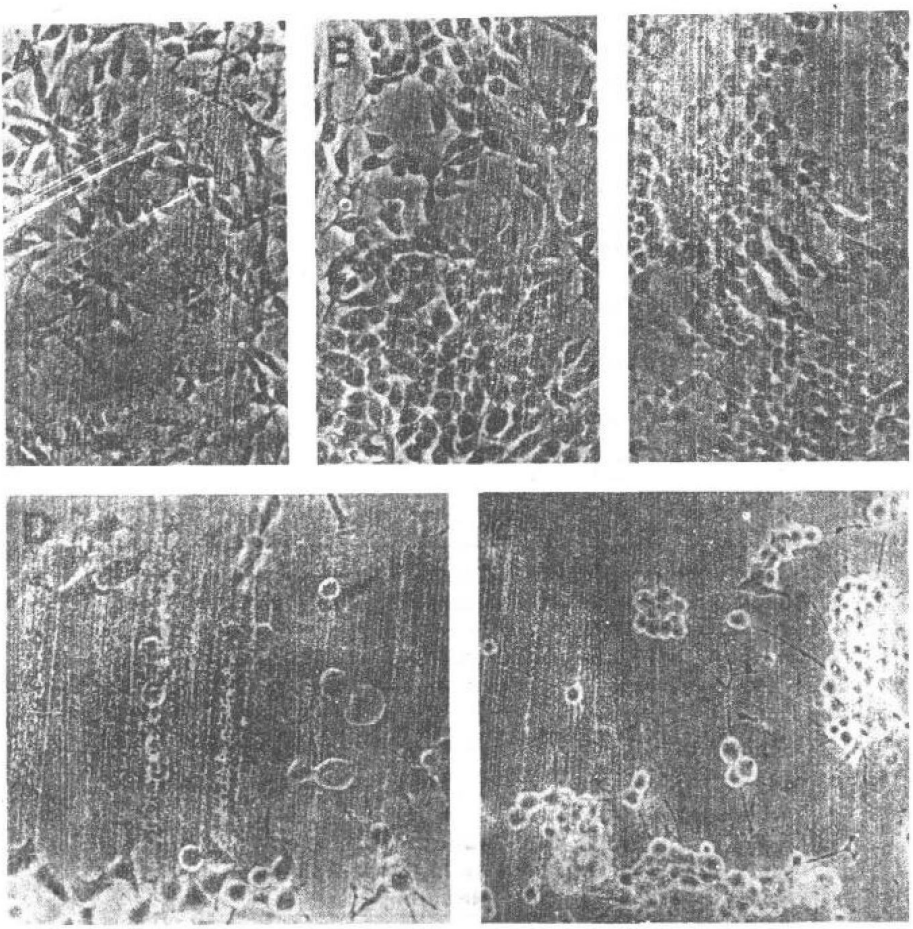

Fig. 1. Morphology of neuronal cell lines grown in N2 medium. A. B104 rat CNS neuroblastoma cells 7 days after plating on a polylysine- and fibronectin-modified substratum. B. LA-N-1 human PNS neuroblastoma cells 6 days after plating on a polylysine- and fibronectin-modified substratum. C. CHP 134 human PNS neuroblastoma cells 5 days after plating on a polylysine- and fibronectin-modified substratum. D. N1E-115 mouse PNS neuroblastoma cells 3 days after switching from serum-containing medium (18 h). E. PC12 rat pheochromocytoma cells 14 days after plating on a fibronectin-modified substratum. Basal medium was a 1:1 mixture of Dulbecco's modified Eagle's (DME) and Ham's F12 medium, except for PC12 cells for which it was DME only.