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Neuronal Differentiation of P19 Embryonal Carcinoma Cells in Defined Media

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The P19 embryonal carcinoma cell line is a useful model system for analyzing the factors that regulate neuronal differentiation. In order to analyze the extrinsic factors that are involved in differentiation, it is necessary to carry out experiments in fully defined media. Here we have investigated the neuronal differentiation of P19 cells in two defined media. Cells that are propagated and induced with retinoic acid in standard serum-containing medium are capable of differentiating into neuron-like cells in N2 medium. Dividing fibroblast-like cells also appeared in these cultures. After about 10 days in culture in N2 medium, the great majority of neuron-like cells died. On the other hand, culturing induced cells in N2 medium for 5 days and then switching to a defined medium consisting of Neurobasal medium plus B27 supplement allowed the neuron-like cells to survive for prolonged periods of time. This defined medium thus provides a suitable system for analyzing extrinsic factors that affect the survival and differentiation of P19 neurons. P19 cells induced with retinoic acid and plated in N2 were exposed to bFGF and EGF, which are known to be mitogens for neuronal precursor cells. Both growth factors were mitogenic for a subpopulation of the induced cells. In separate experiments, cells cultured in N2 in the presence of RA were induced to differentiate into neuron-like cells.

Key words: P19 cells, retinoic acid, neuron differentiation, neuronal cell line, defined media

INTRODUCTION

The P19 embryonal carcinoma cell line is an excellent model system for studying the factors that regulate neuronal differentiation. Under standard cell culture conditions, P19 cells have the distinguishing features of embryonal carcinoma (EC) cells: they are undifferentiated and multipotential, and they divide rapidly. Neuronal differentiation is induced by culturing P19 EC cells under conditions that promote the formation of multicellular aggregates in the presence of retinoic acid for 4 days (Jones-Villeneuve et al., 1982). The induced cells then differentiate into a mixed population of neuron, glia, and fibroblast-like cells. A number of recent studies have established that the neuron-like cells express many characteristic proteins and phenotypes of normal neurons. They are postmitotic, have many neurites, and form structures with the EM appearance of chemical synapses (McBurney et al., 1988). They express the neurotransmitter-synthesizing enzymes cholineacetyltransferase (McBurney et al., 1988) and tyrosine hydroxylase (Sharma and Notter, 1988), the mRNA for both genes coding for glutamic acid decarboxylase (Bain et al., 1993), and glutamic acid decarboxylase protein (Staines et al., 1994). The genes for multiple subunits of ionotropic glutamic acid receptors as well as for functional receptors of the kainate/AMPA and NMDA types are expressed in the neuron-like cells (Ray et al., 1993; Turetsky et al., 1993). Finally, the expression of a number of regulatory genes implicated in neurogenesis, including MASH-1 (Johnson et al., 1992), wint-1 (St. Arnaud et al., 1989; Nusse et al., 1990), and MOTCH (Del Amo et al., 1992), is induced during neuronal differentiation of P19 cells. These and other aspects of P19 neuronal differentiation have recently been reviewed (Bain et al., 1994; McBurney, 1993).

The fact that P19 cells can be induced to recapitulate many features of neuronal differentiation in an in vitro system offers an excellent opportunity to investigate the extrinsic factors that influence or regulate neuronal differentiation. Purified growth factors can be added, to determine if they influence differentiation and if so by what mechanisms. Heterogeneous sources of factors such as tissue extracts or cell-conditioned media can be tested to determine if they have interesting biological activities. Unfortunately, these prospects are diminished by the fact that most of the studies of P19 neuronal differentiation have been performed in serum-

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containing media. The use of serum-containing media presents major obstacles for the analysis of factors which regulate differentiation. First, serum contains a highly complex mixture of metabolites, small molecular weight effectors, peptides, and proteins. Many of these could have profound effects on cells. Therefore, in experiments in serum-containing medium, the investigator has limited knowledge of, and control over, crucial experimental variables. Second, the properties of serum vary according to the species, age, and hormonal status of the donor animal. These variations make reproducibility difficult from lab to lab or within the same lab over time. Finally, serum is inherently unphysiological for the brain. The normal adult brain is separated from serum in the bloodstream by the blood-brain barrier. Thus, neurons maintained in vitro in serum-containing media are exposed to a complex array of molecules which are absent in vivo. The availability of a fully defined medium for culturing P19 cells differentiated to form neurons would overcome these limitations and allow more definitive studies of extrinsic factors involved in differentiation than possible in serum-containing media. Two previous studies suggest that P19 cells can carry out some of their functions in defined media. Schubert and Kimura (1991) discovered that P19 EC cells could be propagated on a fibronectin substrate in N2 medium supplemented with activin A and bFGF. Rates of replication in the defined system were comparable to those in serum-containing medium. However, the developmental potential of cells propagated in this defined medium was not analyzed, so it is not known if they retain the ability to differentiate into neurons. Levine and Flynn (1986) showed that P19 cells induced in standard serum-containing medium could differentiate into neuron-like cells when cultured in the N2-defined medium. While short-term maintenance was demonstrated, long-term culture of neurons was not attempted.

In this study we have further characterized the differentiation of P19 neuronal cells in defined media. A system suitable for long-term, stable culture of these neurons has been discovered. We also show that induction by retinoic acid can take place in a defined medium. In addition, we have characterized the response of retinoic acid-induced P19 cultures to bFGF and EGF, two growth factors that have important effects on embryonic neuronal precursor cells.

MATERIALS AND METHODS

Routine Culture and Induction of P19 Cells

P19 cells were obtained from the American Type Culture Collection (ATCC). They were routinely propagated in α-MEM (GIBCO 12571-014) + 7.5% newborn calf serum + 2.5% fetal bovine serum. Retinoic acid induction of the neural pathway was done by standard methods essentially as detailed in a previous publication from this laboratory (Bain et al., 1993), except that 5% FBS instead of 10% FBS was used in the medium during induction with retinoic acid.

Culture in Defined Media

Dissociation of induced cell aggregates. After aggregates were induced for 4 days they were harvested by sedimentation at 1 *g*, washed once with α-MEM without serum, and resuspended in .25% trypsin-EDTA (GIBCO 15050). DNAase (Sigma D-5025) was added to a final concentration of 50 μg/ml to avoid gel formation by DNA released from damaged cells, and cells were incubated at 37°C for 10 min. At this point, 2 volumes of complete serum-containing growth medium were added to stabilize the cells. Aggregates were then dispersed to single cells by trituration through a serological pipette. The dissociated cell suspension was centrifuged, and the dissociating medium was replaced with N2. The cells were then spun down and the pellet washed once with N2 to remove any traces of serum. Cells were resuspended in fresh N2 medium, counted, and plated in tissue culture wells (9.6 cm^2) with 7.5 × 10^5 cells/well in 2 ml of medium.

Formulation of defined media. N2 medium was formulated according to Bottenstein and Sato (1979). The base medium was a 1:1 mixture of DMEM (GIBCO 11885-043) and Ham's F12 (GIBCO 11765). This was supplemented with insulin, transferrin, selenium, progesterone, and putrescine at the concentrations given in Bottenstein and Sato (1979). Insulin, selenium, and part of the transferrin were from the Sigma ITS mixture (Sigma I-1884). Additional transferrin (Sigma T-7786) was added to a final concentration of 100 μg/ml. Progesterone (Sigma P8783) and putrescine (Sigma P5780) were obtained from Sigma. In some experiments the base medium was Neurobasal medium (GIBCO 21103-015) (Brewer et al., 1993), a medium specifically formulated to optimize survival and development of CNS neurons, to which the B27 supplement was added. For these experiments neurons were first plated in N2 medium as described above and then switched to Neurobasal (NB) medium plus B27 supplement at indicated times.

Cell attachment factors. Poly L-lysine (Sigma P2636) was dissolved in borate buffer, pH 8.2, at 0.1 mg/ml. This solution was added to wells overnight. They were then washed extensively with 1 × PBS. Bovine serum-derived fibronectin (FN) (Sigma F-1141) was used to coat wells. Five μg of fibronectin in 0.5 ml DMEM were added to each well and incubated overnight. Wells were then washed with 1 × phosphate-buffered saline (PBS).

Propagation of EC Cells in Defined Medium

Cells were propagated in defined medium essentially as described by Schubert and Kimura (1991). Medium consisted of N2 + bFGF (10 ng/ml) + activin A (12 ng/ml); cells were plated on FN.

Growth Factors

The following growth factors were purchased: human recombinant bFGF (GIBCO 3256SA), human recombinant EGF (Sigma E-1264), leukemia inhibitory factor (LIF) (GIBCO-BRL 3275 SA; ESGRO), and B27 supplement (GIBCO 17504-010).

Antibodies

The antibodies used in this study and their sources are: anti-SSEA-1 (Developmental Studies Hybridoma Bank); anti-class III-β microtubule protein designated *Tuj*1 (Dr. Anthony Frankfurter); anti-neurofilament designated RMO 108 (Dr. V. Lee); anti-neurofilament M designated 3H11 (Dr. Gerry Shaw).

Staining With DiI

Cultures were fixed with 2% paraformaldehyde and 1% glutaraldehyde in 1 × PBS, pH 7.4, for 1 hr at room temperature. Fixative was washed off and replaced by 1 ml PBS containing DiI (Molecular Probes, Inc.) at a final concentration of 12.5 μg/ml, and stained for 1 hr. Cultures were washed with PBS to remove unbound dye and photographed with fluorescence optics using rhodamine filters.

RNase Protection Assays

Whole cell RNA was prepared as described (Bain and Gottlieb, 1994) from undifferentiated P19 cells, P19 cells differentiated for 6 days under standard conditions in serum-containing medium, or from differentiated P19 cells cultured in NB + B27 serum-free medium for 17 days. Expression of GAD_{67} mRNA was detected by an RNase protection assay as described (Bain et al., 1993), using 50 μg of RNA and 5 × 10^5 cpm of radiolabelled probe.

RESULTS

In these experiments, the use of defined medium for each of the major steps of P19 culture was explored. In the first group, cells were both propagated and induced in standard serum-containing medium; after induction they were plated in defined media. In the second group, propagation was in serum-containing medium followed by both induction and plating in defined medium. In the last set, propagation, induction, and plating of induced cells were in defined medium.

Propagation and Induction in Serum-Containing Medium: Plating in Defined Media

For these experiments, cells were propagated under standard conditions in α-MEM with serum as detailed in Materials and Methods. Induction was by the standard retinoic acid procedure in α-MEM with 5% FBS. The objective of these experiments was to observe the differentiation of induced cells in N2 medium on 3 substrates that are commonly used for primary culture of cells from the nervous system: tissue culture plastic (TCP), fibronectin (FN), and poly-L-lysine (PL). Aggregates of induced cells were dissociated with trypsin, and cell suspensions were plated onto each of these substrates. Cultures showed significant differences among the 3 substrates; therefore each type is described separately.

Growth on tissue culture plastic (TCP). Figure 1A,B illustrates some features of these cultures. Shortly after plating, the cells begin to adhere to the plastic. By day 2, the majority of cells are small and phase-dark. Some cells have sprouted processes of up to 10 cell diameters in length, and often have well-formed growth cones at their ends. These cells strongly resemble the immature neurons seen in dissociated cultures of the early brain. Some of the small phase-dark round cells are found in clusters. It is not clear if the clusters were present at the time of dissociation, came about by aggregation, or arose by cellular division. There are also a small number of very flattened fibroblast-like cells. Processes from the small phase-dark cells have a tendency to grow on the surface of these fibroblast-like cells. By day 5, the appearance of the cultures has changed considerably. Most of the neuron-like cells are now in multicellular clusters. The clusters are roughly spherical, with their bottoms attached to the substrate. Clusters typically give off processes which extend several hundred micra. Some of the processes are much thicker than individual axons and so must consist of fasciculated axons. Processes are strongly attached at their tips to either the dish or flat cells, and the trailing portion is frequently suspended in the medium. Few if any individual neurons are found by day 5; instead, the neurons are in clusters. By day 5, the population of flattened fibroblast-like cells has increased relative to 2-day cultures. These cells continue to be a substrate for processes from the neurons. The neurons in cultures on tissue culture plastic become mechanically unstable after about 1 week. Neural clusters lose their attachment and float free. There is also substantial cell degeneration in the clusters. In contrast to the neurons, background fibroblast-like cells are stable and can be cultured for longer periods of time.

Growth on fibronectin (FN). Features of cultures on a fibronectin substrate are illustrated in Figure 2A,B. The cultures are similar to those on untreated tissue cul-

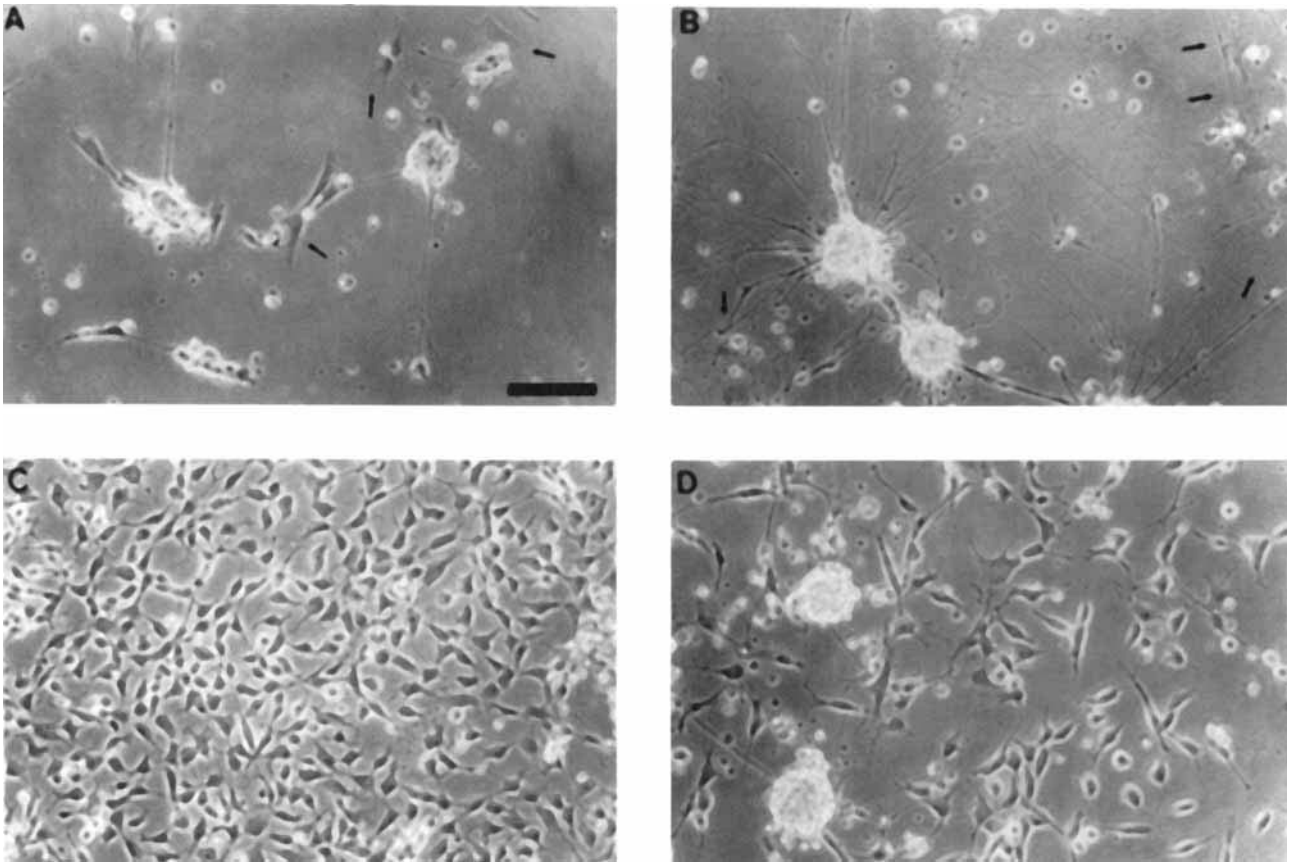

Fig. 1. Growth of retinoic acid-induced P19 cells in N2 medium on untreated tissue culture plastic. Cells were induced with retinoic acid, dispersed, and plated in N2 medium with indicated additions as described in the text. Photographs show the appearance of cultures at indicated day postplating. Bar in all photographs, 100 microns. **A:** 2 days. Note clumps of cells with neurites coming out; the neurites appear to be fasciculated. Arrows point to flat background cells. **B:** 5 days. Aggregates of neurons now give off multiple neurites. Background cells (arrows) are more numerous. **C:** 5 days + bFGF. Note the presence of many attached, small polygonal cells not present in culture without growth factor and which now dominate the culture. **D:** 5 days + EGF. Clumps of small cells with neurites remain. Many small polygonal cells not present in culture without growth factor are present.

ture plastic but have several significant differences. Within a day after plating, the cells attach firmly to the substrate and are more spread out than cells on untreated plastic. By day 2, the cultures consist of small phase-dark cells, many with neurites, and of the large fibroblast-like background cells described for control cultures. All of the cells are more spread out on the substrate, and process formation appears to be accelerated relative to cultures on plastic alone. Over the next 2 days, extensive process formation occurs. While there is a tendency to form clusters as in controls, it is less pronounced in these cultures than on untreated tissue culture plastic. Cultures contain many spread-out, single neurons, even at day 4 and 5 postplating, as well as multicellular clusters. Taken together, the observations of these cultures during the first 5 days postplating suggest strongly that newly differentiated P19 neurons have functional receptors for FN. Upon further culture all neurons do join clusters. These clusters give off large fasciculated bundles of neurites, most of which are attached only at their tips. By day 7, cultures are mechanically unstable and clusters of neurons detach from the surface.

Cultures on FN have the large, tightly adherent fibroblast-like cells seen in cultures on untreated tissue culture plastic. On FN their number increases more rapidly than controls. By day 5, most of the surface area is covered by a monolayer of these cells. Proliferation stops after the formation of a monolayer, presumably due to a density-dependent inhibitory mechanism.

Growth on poly-L-lysine. Features of these cultures are illustrated in Figure 3A,B. At 2 days postplating most of the cells are small, phase-dark, and firmly attached to the substratum. A significant fraction have neuritic processes. There are fewer well-spread fibro-

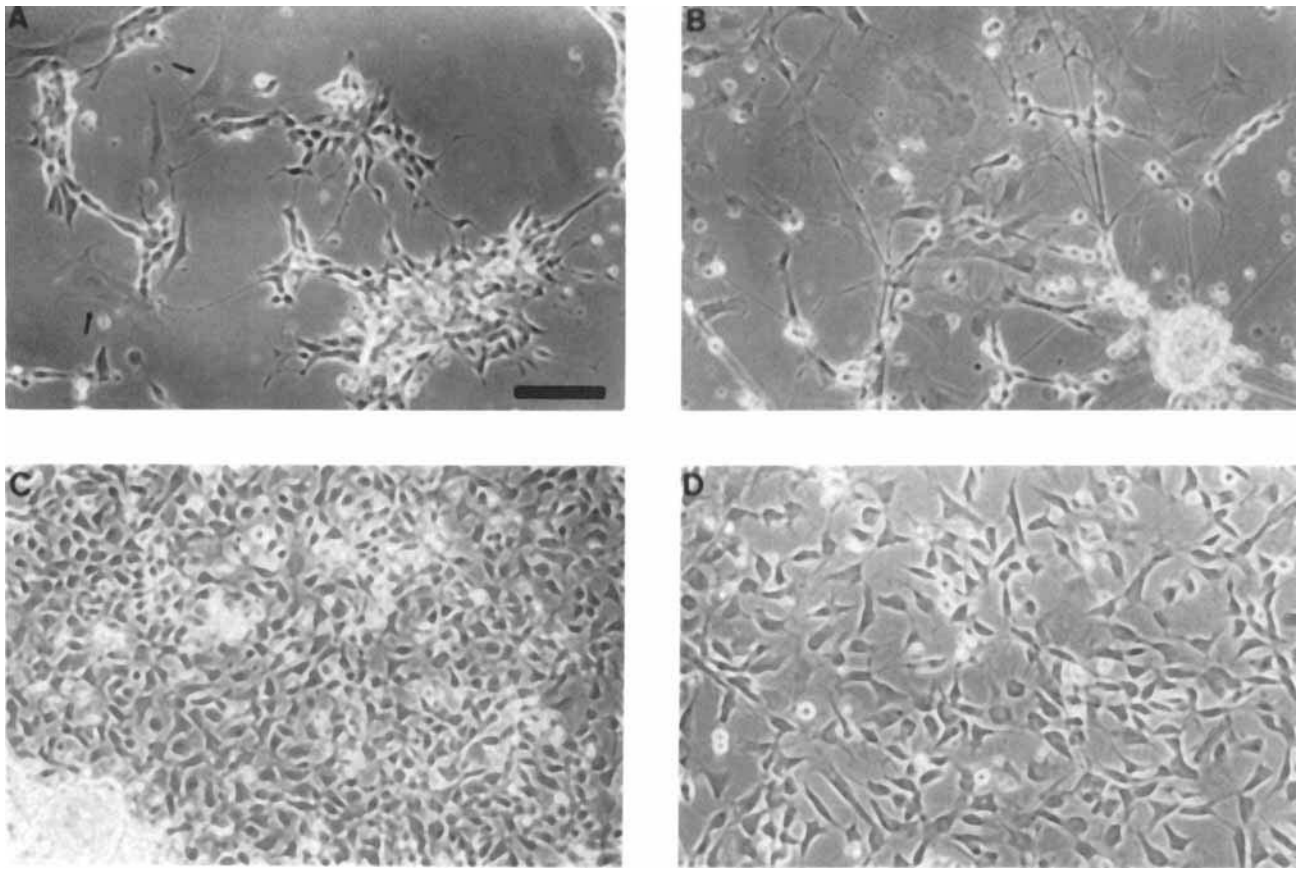

Fig. 2. Growth in N2 medium on fibronectin (FN) substrate. Cells were induced with retinoic acid, dispersed, and plated on a fibronectin-coated substrate in N2 medium with indicated growth factors. Photographs show appearance of cultures on indicated day postplating. **A:** 2 days. Most of the neurons are spread out and isolated, compared to cells on untreated plastic; neurite formation is extensive. Flat background cells are indicated by arrows. **B:** 5 days. Neurons are a mix of aggregates and spread cells. Note the increase in the number of background cells. **C:** 5 days + bFGF. Note extreme proliferation of small polygonal cells. **D:** 5 days + EGF. Note proliferation of small polygonal cells.

blast-like flat cells than on TCP or FN, a characteristic that continues throughout the life of these cultures. From days 2–4 the neurons join clusters. Each cluster gives off many neurites. Some of the neurites are of very small diameter and are likely to be single neurites that have not fasciculated with their neighbors; other neurites are found in fascicles.

Unlike cultures on TCP and FN, the PL cultures are mechanically stable and can therefore be studied for longer periods of time than cultures on TCP and FN. They are also suitable for procedures that require multiple washes, such as antibody staining. Neurons in cultures on a PL substrate continue to appear healthy until about day 8 postplating. At this point a major wave of neuronal cell death sweeps over the cultures and most of the cells in the clusters die. Fibroblast-like background cells remain viable, indicating that the cultures are not generally toxic. While there is some variability from experiment to experiment as to exactly when neuronal cell death occurs, we find that all PL cultures show extensive neuronal cell death within the first 12 days. Successful efforts to maintain cultures for longer in a defined medium are described below.

Cells with neurites express neural specific antigens. The phase appearance of the process-bearing cells in Figures 1–3 is highly consistent with the hypothesis that they are neurons. To confirm this they were stained with antibodies to neuron-specific class III-β tubulin (antibody *Tuj*1) and neurofilament proteins (antibodies ROM108 and 3H11). We attempted to stain cultures grown on all 3 substrates. Unfortunately, neurons on TCP and FN substrates detached from the dishes during the staining procedure and could not be analyzed. Cultures on PL were stable enough to withstand the antibody staining procedure. Figure 4 shows that these antibodies stain neuron-like cells on PL substrate 4 days after plat-

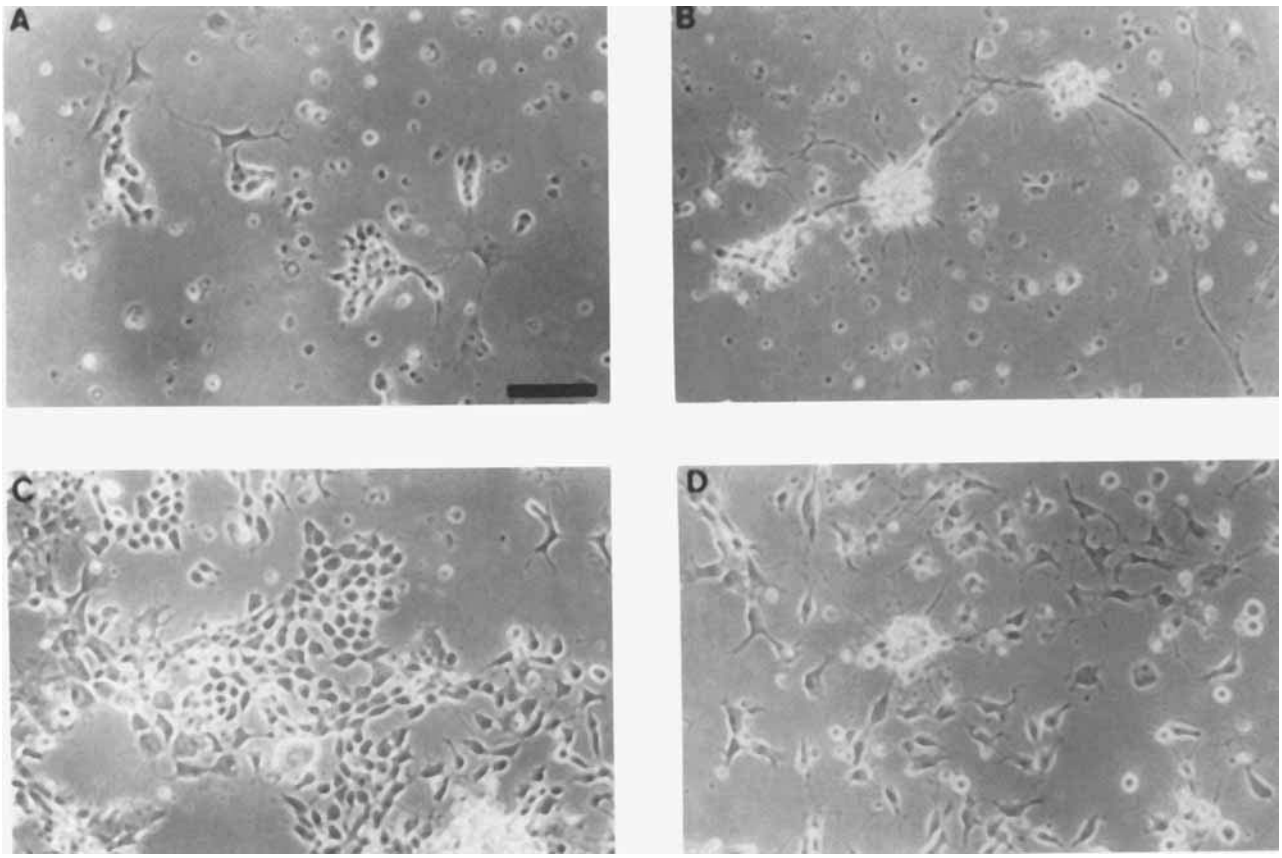

Fig. 3. Growth in N2 medium on a poly-L-lysine substrate. Cells were induced with retinoic acid, dispersed, and plated on a poly-L-lysine-coated substrate in N2 medium with indicated growth factors. Photographs show appearance of cultures on indicated day postplating. **A:** 2 days. Many cells are small, round, and phase-dark, and are arranged in 2-dimensional islands. Some have short neurites emerging. There are a few large, spread background cells. **B:** 5 days. Neurons are present mostly in 3-dimensional aggregates. Extensive arrays of neurites, most of them tightly adherent to the substrate and not fasciculated, are given off by the aggregates. **C:** 5 days + bFGF. Note presence of many small polygonal cells not seen without the growth factor. Comparison with Figures 1C and 2C suggests that proliferation of these cells occurs on poly-L-lysine, but more slowly than on other substrates. **D:** 5 days + EGF. Note presence of many small polygonal cells. There are fewer of these than on other substrates. Note also that aggregates with neurites are still present.

ing. We conclude that 2 proteins highly characteristic of neurons are expressed by neuronal cells differentiating in N2 on a PL substrate.

Effect of bFGF and EGF. Both bFGF and EGF have been shown to be mitogens for neuronal precursor cells in the developing nervous system. Therefore, experiments were done to determine if they might be mitogenic for P19 cells that had been induced to differentiate along the neuronal pathway. When bFGF is added to cultures in N2 at the time of plating, cultures appear indistinguishable from controls for the first few days. Then a population of mitogenic cells appears. This happens on TCP, PL, or FN substrates, but the effect is more rapid and extensive on FN (Figs. 1C, 2C, 3C). The proliferating cells are small, polygonal, and phase-dark. Their proliferation is not blocked by density-dependent inhibition, and they form a multilayer of cells. Usually the cells which proliferate in response to bFGF are so numerous that they dominate the culture. Therefore, it is difficult to determine if neurons remain in cultures treated with bFGF. Since bFGF is mitogenic for P19 EC cells (Schubert and Kimura, 1991), one possibility is that the cells which proliferate in response to bFGF in induced cultures are EC cells that "escaped" induction. However, this appears unlikely, since the vast majority of these cells do not stain with an antibody to SSEA-1, an antigen expressed strongly by P19 EC cells (Fig. 5).

Experiments were also performed in which EGF was included at the time of plating for cultures on all 3 substrates. This resulted in the appearance of small polygonal cells that were not present in control cultures without added growth factor. On TCP there is extensive

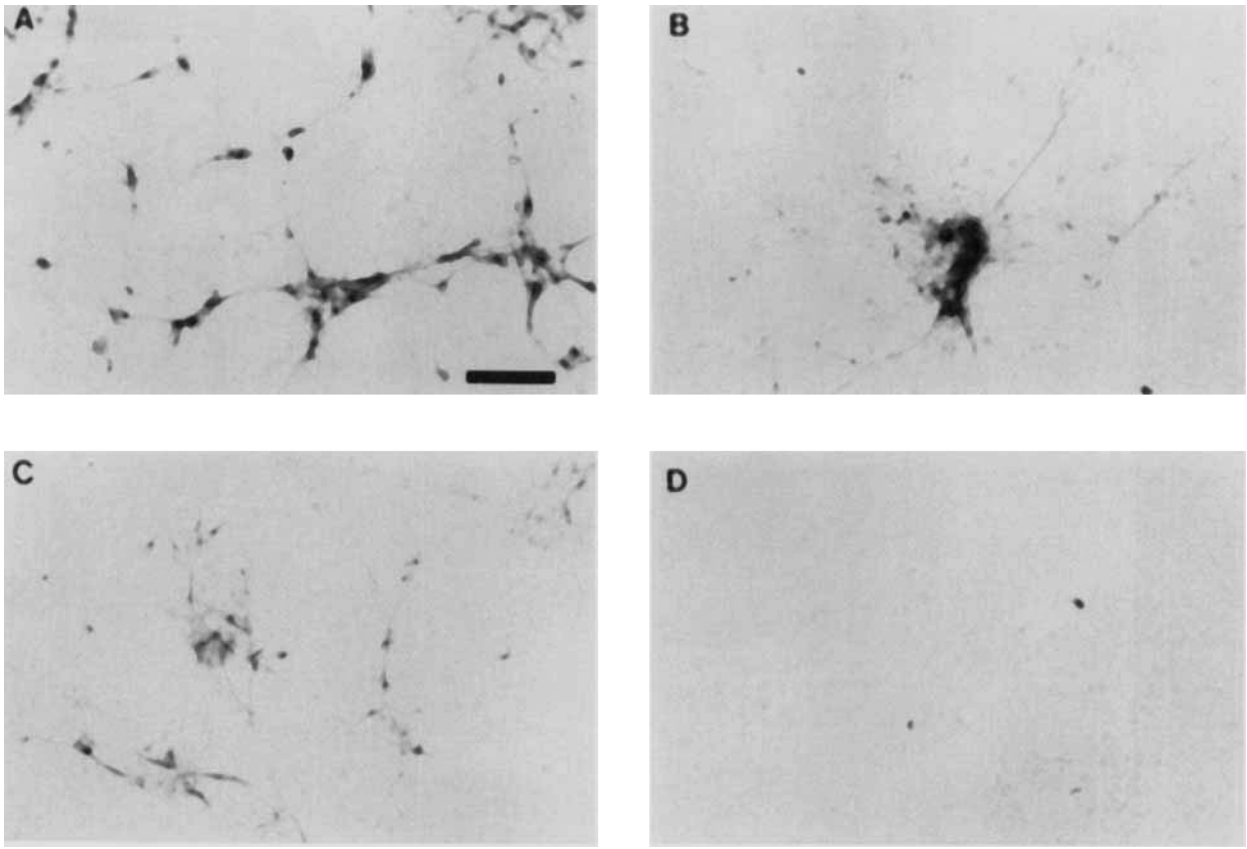

Fig. 4. P19 cells maintained in N2 express neural antigens. P19 cells were propagated and induced under standard conditions. They were then plated onto a PL substrate in N2 medium for 4 days, fixed, and stained. **A:** Primary antibody *Tuj*1 against neuron-specific microtubule subunit. **B:** Primary antibody ROM-108 against neurofilament. **C:** Primary antibody 3H11 against neurofilament subunit M. **D:** Normal mouse serum control. Note that cells are present containing each of these antigens; also note lack of staining in the control.

proliferation of such cells by day 5. On FN in the presence of EGF, there was a vigorous proliferation of cells within 2–3 days. By day 5, these formed a multilayer and had crowded out the normal neurons. On PL, this class of cell also appeared in response to EGF but the rate of its appearance was lower than on the other substrates. These results are shown in Figures 1D, 2D, and 3D. The cells that proliferate in response to EGF do not stain with an antibody to SSEA-1 (Fig. 5), indicating that they are not a residual population of uninduced EC cells.

Growth in Neurobasal medium + B27 supplement. As indicated above, neurons in cultures maintained in N2 medium on a PL substrate appear healthy and grow well for about the first 8 days after plating. They then undergo very extensive cell death. This could be due to lack of essential factors in the N2-defined medium. Alternatively, it might be due to factors in N2 medium that are not toxic to young neurons, but are to older neurons. Glutamic acid is a likely candidate for such a toxic factor, since P19 neurons are initially unresponsive to glutamate but later express functional receptors for this neurotransmitter, the activation of which leads to excitotoxic cell death (Turetsky et al., 1993). Thus, while N2 is a suitable medium for studying the *initial* phases of neuronal differentiation, it is not permissive for later development. In order to do experiments on P19 neuronal maturation past the initial phase in defined medium, it is essential to find a way to prevent this degeneration and to have cells remain viable for extended periods. Recently, a new defined culture medium has been formulated which is suitable for the long-term culture of hippocampal neurons (Brewer et al., 1993). It consists of a defined medium (Neurobasal medium; NB) and a defined supplement, designated the B27 supplement. This new, fully defined formulation was tried on P19 cultures. Cultures of differentiated cells in

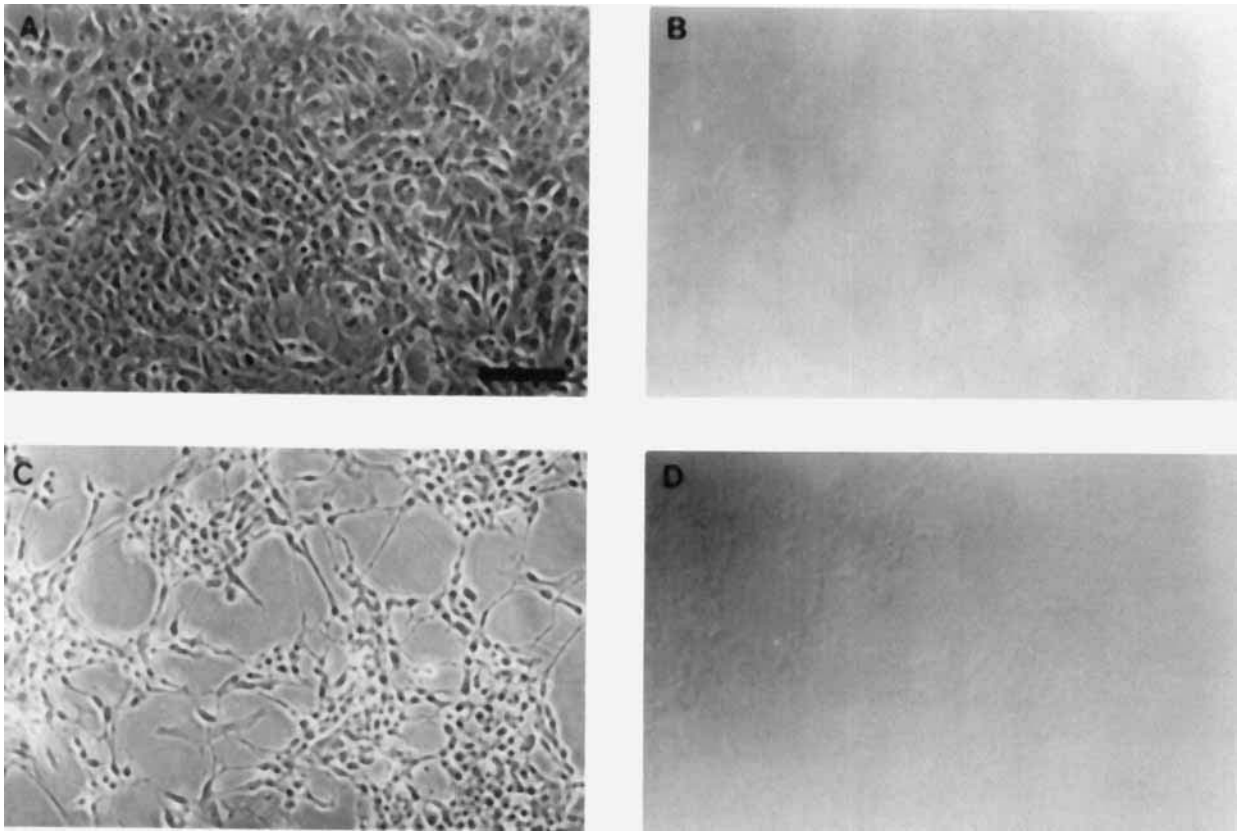

Fig. 5. Flat cells that divide in response to bFGF and EGF are SSEA-1-negative. P19 cells were induced by the standard method and plated on FN in N2 medium with indicated growth factor for 3 days. They were fixed and stained with SSEA-1 antibody. P19 EC cells stain intensely with this antibody (data not shown). **A:** bFGF, phase contrast optics. **B:** bFGF fluorescence optics. **C:** EGF phase contrast optics. **D:** EGF fluorescence optics. Bar, 100 microns.

N2 medium on a PL substratum were set up as described above. On day 6 postplating they were switched from N2 to Neurobasal medium + B27. Cultures appeared comparable to controls maintained in N2 until 8–10 days. At this point a marked difference was apparent. Cultures in N2 medium began to show extensive signs of degeneration. By about day 12, few healthy axons or neuronal cell bodies were left in the cultures in N2. In contrast, cultures switched into NB + B27 remained healthy. Neurites continued to grow and went on to form an extremely dense network. Cell bodies remained healthy-looking. These cultures remained healthy for 3 weeks (Fig. 6). At this time the vast majority of neurons in N2 cultures had degenerated (Fig. 6). We conclude that the extensive cell death that occurs in N2 alone is prevented by culture in the Neurobasal medium + B27 formulation.

Differentiated P19 cells in standard serum-containing media express a variety of characteristic neuronal proteins (reviewed in Bain et al., 1994). GAD_{67} is one of the genes highly expressed. In order to show that at least one gene is highly expressed in P19 cells maintained in NB + B27, levels of GAD_{67} transcripts were assayed. The results show that high levels of GAD_{67} transcripts are present in cells maintained for 17 days in NB + B27 (Fig. 7).

Induction by RA in a Defined Medium

The results presented above demonstrate that P19 cells which are propagated and induced in serum-containing media are capable of extensive differentiation when subsequently plated in defined media. Currently, there is a great deal of interest in mechanisms that are active during induction with retinoic acid itself. Numerous molecular changes have been shown to occur during this phase (reviewed in Bain et al., 1994). In order to investigate the role played by extrinsic factors in the induction process it would be desirable to culture the cells in as simple and defined a medium as possible.

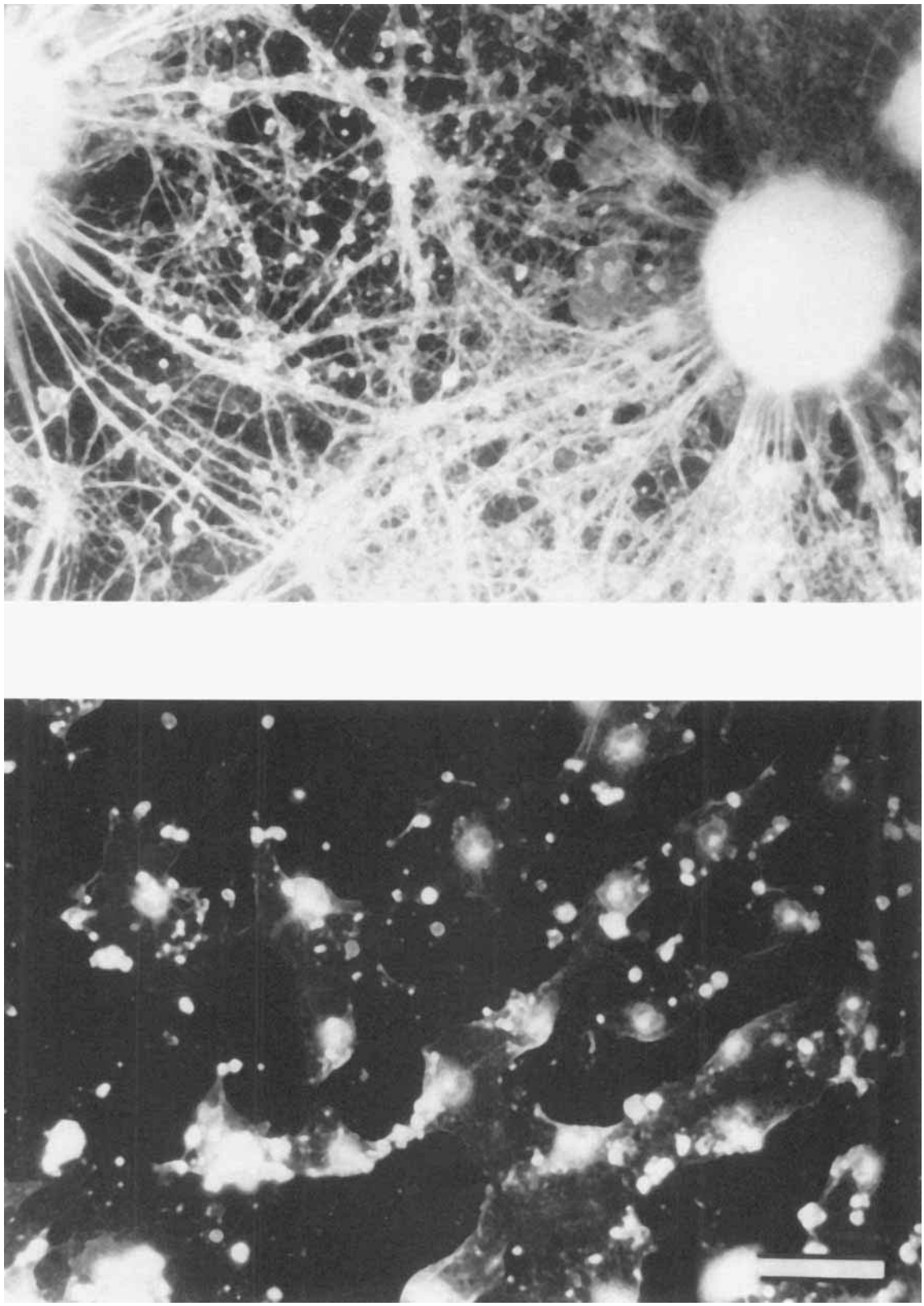

Fig. 6. NB medium + B27 supplement promotes survival of P19 neurons. P19 EC cells were induced by the standard method and plated in N2 on PL. At 6 days postplating, the medium in wells was either replaced with N2 (**bottom**) or with NB + B27 (**top**). Cultures were fed at 4–5 day intervals with the same medium. After a total of 21 days in culture postplating, cells were fixed and stained with DiI. Representative fields are photographed. Note the much greater extent of axons in the cultures in NB + B27. Bar, 100 microns.

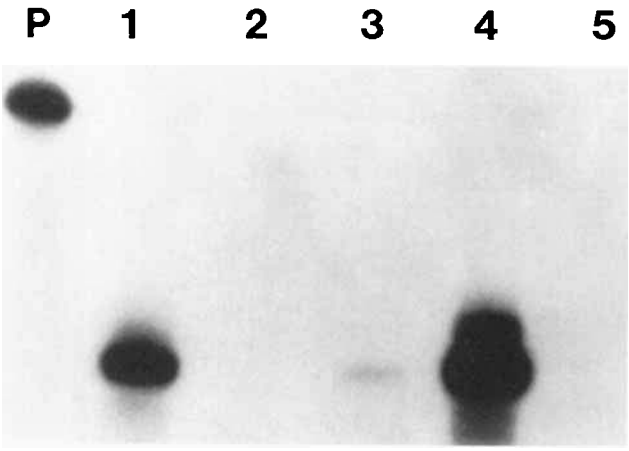

Fig. 7. Expression of GAD_{67} transcripts. The expression of GAD_{67} gene transcripts in P19 cells cultured under several conditions was assayed by RNase protection assay. RNA from adult mouse brain (5 μg; **lane 1**) is a positive control, and yeast tRNA (50 μg; **lane 5**) is a negative control. Whole cell RNA (50 μg) from undifferentiated P19 stem cells (**lane 2**), differentiated P19 cells cultured under standard conditions in serum-containing medium for 6 days after dissociation of RA-treated aggregates (**lane 3**), and differentiated P19 cells cultured in NB + B27 serum-free medium for 17 days after dissociation of RA-treated aggregates (**lane 4**) were assayed. The radiolabelled probe is shown in **lane P.**

Therefore, we investigated the response of P19 EC cells propagated in serum-containing medium to induction by RA in N2. EC cells propagated in standard medium were removed from their flasks by standard trypsin treatment and switched into N2 medium with retinoic acid. These cell suspensions were plated in the same type of bacteriological culture dishes used for standard inductions, and cultured for 4 days. In some cases cultures were also supplemented with leukemia inhibitory factor (LIF) at .01 μg/ml. Initially, the majority of cells adhered firmly to the substrate; this is in marked contrast to serum-containing cultures which do not adhere to bacteriological dishes. Upon further culture the cells resolved into a mixture of small 3-dimensional groups of cells, 2-dimensional cell clusters, and isolated cells. There is a tendency for cultures with LIF to contain fewer 3-dimensional aggregates, but this was not studied quantitatively. While there was a variable degree of cell death in these cultures, the majority of cells survived the 4-day induction period. LIF seemed to improve survival slightly.

Cells induced in N2 and retinoic acid were trypsinized and plated in N2 on an FN substrate. These cultures showed extensive neuronal differentiation (Fig. 8). Most features of these cultures paralleled those of cultures induced in standard serum-containing media. These cultures were maintained for only 6 days, and their response to culture in Neurobasal medium was not investigated.

Propagation, Induction, and Differentiation in Defined Media

Finally, an experiment was performed to determine if all aspects of culture could be carried out in defined medium. Cells were propagated in a defined medium consisting of N2 + activin A + bFGF on an FN substrate for 5 days, and induced in N2 + LIF + RA for 4 days. They were then dispersed and plated in N2. A few neurons were formed, but they were far fewer than in cultures propagated and induced by standard methods. We conclude that propagation of cells in defined media somehow reduces their ability to differentiate.

DISCUSSION

The pathway of differentiation from P19 EC cells to neurons induced by retinoic acid is a suitable model for detailed investigation of the mechanisms of neuronal differentiation. A desirable precondition for an extensive analysis of this pathway, particularly for the investigation of extrinsic factors that influence differentiation, is a defined and simplified culture system. The results of this study provide substantial steps in that direction. They show, in agreement with the findings of Levine and Flynn (1986), that P19 cells which are propagated under standard conditions and then induced with retinoic acid in standard serum-containing medium can differentiate into neurons in N2 medium. Freshly-plated induced cells which are small and round, and which lack processes, attach to 3 commonly used tissue culture substrates, TCP, FN, and PL. Many of the cells extend neurites and develop the morphology characteristic of mammalian neurons in primary culture. In addition to a neuronal morphology, these cells express class III-β microtubule protein and neurofilament proteins. Neuritic development is very extensive, so that by about day 7, cultures consist of aggregates of cells connected by long, thick bundles of neurites. The regulatory events responsible for controlling the differentiation of newly induced cells into neurons are largely unknown, although a few proteins have been directly implicated as playing necessary roles (Bain et al., 1994). The results of this study show that the overall process does not depend on factors present in serum. One interpretation of the results is that early differentiation after induction is completely independent of extrinsic regulatory factors other than perhaps insulin, which is present in N2. Another possible interpretation is that extrinsic growth factors or regulatory factors are important, but that they are supplied by the P19 cells themselves. Factors secreted by the P19 neurons themselves or by the flat dividing cells which are

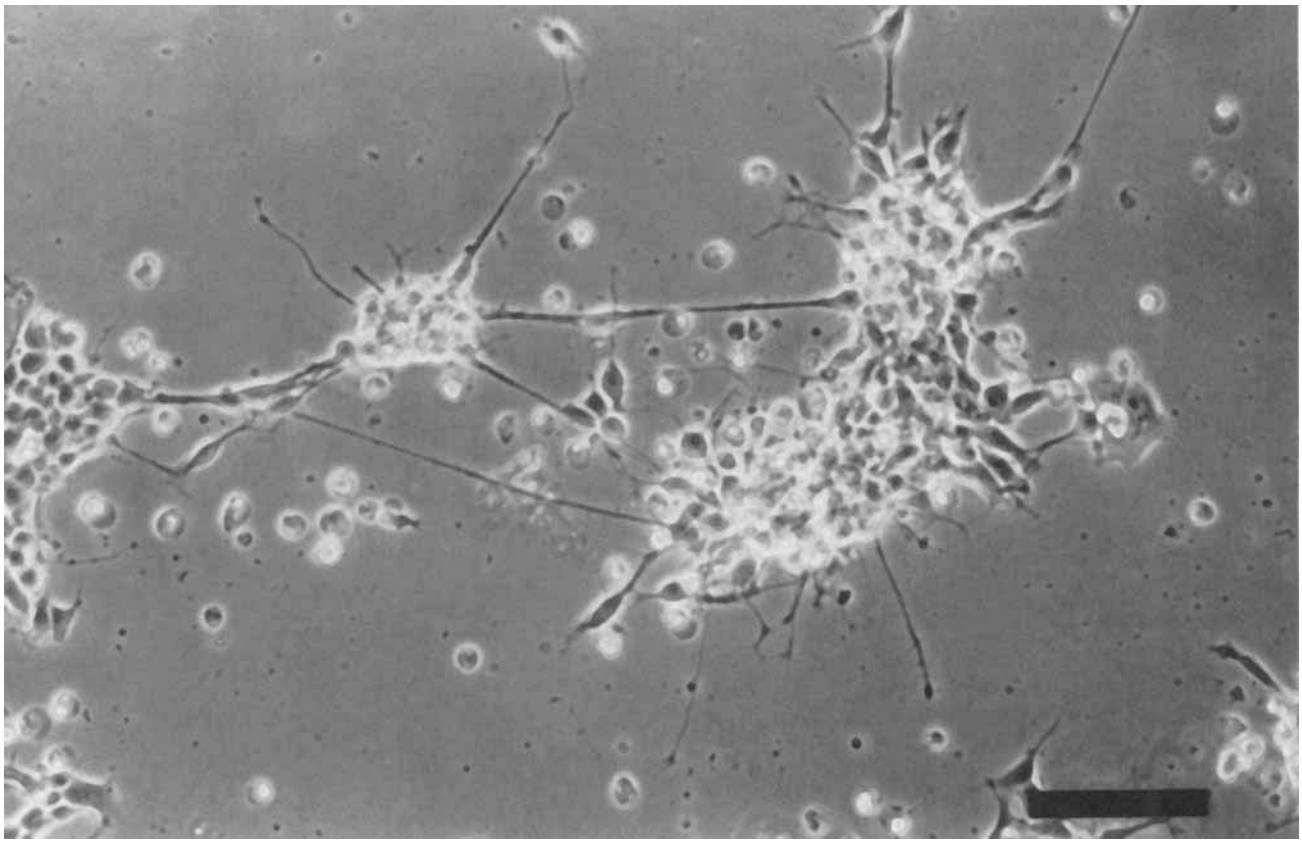

Fig. 8. Differentiation of P19 cells propagated in serum-containing medium and induced in N2. Induction was in N2 + RA + LIF as described in Results. After 4 days of induction, the cells were trypsinized and plated on an FN substrate. Cultures were photographed 3 days after plating. Note the numerous neurites. Bar, 100 microns.

present in these cultures may be necessary for neuronal differentiation. Alternatively, since many cells are in contact with their neighbors, regulatory molecules found on the cell surface may be crucial. Additional investigation is necessary to determine which of these possibilities is actually true. The results also show that the substratum used for culture has an important influence on the differentiation of neurons. In particular, neurons cultured in N2 on fibronectin interact strongly with the substratum. They extend neurites more rapidly and remain as individual, well-spread cells longer than cells on either TCP or PL. The contrast between FN cultures and TCP cultures suggests that newly differentiated neurons have functional FN receptors. The fact that the $\alpha_v\beta_1$ type integrin subunits are upregulated during differentiation (Dedhar et al., 1991) is consistent with this idea. However, the fact that neurons develop very well on TCP shows that interaction with FN is not *essential* for neurite growth and development. This is a novel conclusion from these studies, as Levine and Flynn (1986) utilized FN in their cultures, and other studies on P19 neuronal differentiation have been done in serum-containing medium which contains FN that is capable of coating the substrate.

While N2 medium is sufficient to support the early differentiation of P19 neurons, it is clearly not suitable for prolonged culture. After about 8–10 days of culture in N2, the majority of neurons degenerated rapidly. This is easiest to observe in cultures on the PL substrate since they are mechanically stable, but the same can also be seen in cultures on TCP or FN. The onset of neuronal cell death places a severe limitation on the types of experiments which can be done in N2 medium. Cultures in N2 medium are clearly robust and healthy for about 6 days after plating of newly induced cells because the extent of the neurite network in these cultures increases rapidly. Dramatic and pervasive death of neurons is evident by day 10. Therefore, sometime in the interval of 6–10 days postplating, the typical neuron in the cultures stops being healthy and begins to degenerate. Neural death could be due to toxic factors in the N2 medium. Glutamic acid, a component of N2 medium, is a clear candidate for a toxic

component since P19 neurons develop glutamate receptors and are subject to toxicity by activation of these receptors (Turetsky et al., 1993). Alternatively, death of neurons in N2 may be due to a deficiency of one or more components in N2 medium. In either case, the consistent death of neurons after about 10 days in culture in this medium limits the usefulness of N2 as a defined medium for analyzing differentiation of P19 neurons. Any analysis in N2 must be limited to the first 5 or 6 days when the cells are healthy and growing. Experiments at later times in N2 will be confounded by death of the neurons. Neuron death can consistently be prevented by switching cells from N2 into another defined medium, Neurobasal medium + B27 supplement (Brewer et al., 1993). This formulation is more complex than N2 but is still fully defined. In this medium, P19 neurons consistently continue to survive past 10 days. They appear healthy under microscopic observation and maintain very dense networks of processes on the substratum. Culture in Neurobasal medium + B27 is thus a suitable system for future experiments analyzing factors that affect the development and maintenance of P19 neurons in a fully defined medium. It is not clear from the present results why the new formulation is effective at maintaining P19 neurons in prolonged culture. The formulation lacks glutamate and aspartate and thus would avoid the toxicity of these components. The B27 supplement contains the components that allow N2 medium to substitute for serum (insulin, transferrin, selenium, putrescine, and progesterone), but has additional components. Perhaps these contribute to the beneficial effects of this medium. An additional feature of cultures in the new formulation is that the flat background cells are greatly diminished. The culture system thus offers an opportunity to analyze P19 neurons in relative absence of background cells.

In addition to neurons, cells induced by retinoic acid in serum-containing medium give rise to flat adherent cells with the capacity to divide, some of which have properties of glial cells (Jones-Villeneuve et al., 1982). In the present studies, when induced cells are plated in N2 medium, a class of nonneuronal cells attaches to the substrate and becomes a prominent part of the cultures. These cells have a very different morphology from EC cells and therefore clearly are not simply EC cells that failed to respond to retinoic acid. Cultures on FN substrate contain more of these cells than cultures on TCP or PL, and their numbers increase with time in culture. The question of whether these cells divide in response to a mitogen and, if so, what is its source, is intriguing. N2 medium contains levels of insulin high enough to activate IGF receptors; these might exert a mitogenic action. Alternatively, mitogens might be supplied by the flat cells themselves in an autocrine fashion. Finally, mitogens may be supplied by the P19 neurons since it is known that neurons can produce mitogens active on other cell types.

bFGF added to newly-plated cultures in N2 has a very strong mitogenic action. A population of small, polygonal, rapidly dividing cells rapidly overtakes these cultures. These cells resemble EC cells in morphology but are clearly distinct from EC cells since they do not express the SSEA-1 cell surface antigen. They have not been characterized further. EGF is also a mitogen for a population of cells in induced cultures in N2. Like the cells that divide in response to bFGF, these cells do not express the SSEA-1 antigen and hence are not EC cells. This conclusion is reinforced by the fact that P19 EC cells do not divide in response to EGF (Schubert and Kimura, 1991; Yao and Gottlieb, unpublished observations). The nature of the cells which divide in response to bFGF and EGF is an intriguing problem. These factors are mitogenic to neural precursor cells (Anchan et al., 1991; Lillien and Cepko, 1992; Ray et al., 1993; Reynolds and Weiss, 1992). Perhaps P19 cells that divide in response to bFGF and EGF have properties of neuronal precursors. Otherwise, they might be cells destined for an alternative developmental fate.

A number of important regulatory events leading to the neuronal phenotype occur during the 4-day period of induction with retinoic acid (reviewed in Bain et al., 1994). A deeper analysis of this critical phase of differentiation will greatly benefit from culture in defined medium. Our results show that significant differentiation can be obtained in cells exposed to retinoic acid while cultured in N2 medium. The results establish that at least some of the critical processes of induction can take place in the absence of serum. However, it is important to note that such an interpretation of this result has two limitations. Although the cells are cultured without serum, it is still possible that regulatory factors such as receptors, second messengers, or transcription factors that are induced by serum still persist in cells undergoing retinoic acid induction in serum-free medium. The observation that cells both propagated and induced in defined media differentiate poorly is consistent with this idea. A second limitation is that we have only observed the morphology of these cells. While they appear neuronal, it is conceivable that some of the neuronal phenotypes found in cells induced in serum-containing media are lacking.

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