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Method for preparing cultures of central neurons: Cytochemical and immunochemical studies

(neuroblasts/astrocytes/oligodendrocytes/glial fibrillary acidic protein/insulin)

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ABSTRACT **We report a simplified method for culturing fetal central nervous system cells predominantly inducing neurons that grow, differentiate, and live *in vitro* for as long as 10 weeks. These central nervous system cells form a confluent cell culture in which about 80% of the cells are fully differentiated neurons producing interconnecting axons and dendrite processes and live upon a sparse underlying population of fibrillary and protoplasmic astrocytes, oligodendrocytes, and fibroblasts. Morphological and cytochemical characteristics of these cell types, based on immunofluorescent cell specific markers and silver staining of neurons, are presented.**

Large numbers of neurons can be cultured from dissociated fetal brain of several species of rodents by the technique described in this paper. The growth of central neurons is relatively unencumbered by the concurrent growth of fibroblasts or glial cells, and the fine structure of the neurons can be readily studied by light and electron microscopy. Neurons are easily identified by their cytological structure and their specific cytochemical properties and may be distinguished from the main classes of other cells growing together with them by the use of cell-specific immunofluorescent markers.

The method for readily culturing large numbers of neurons from the fetal central nervous system is based on many essential modifications of other methods used for isolation, growth, and maintenance of central neurons in culture for long periods of time (1–8). There are three major modifications: (*i*) use of a fetus chosen at a critical phase of early development—at the beginning of organogenesis—while there are many still dividing neuroblasts that possess ability to survive under tissue culture conditions and to differentiate into mature neurons; (*ii*) use of arabinosylcytosine, a specific inhibitor of DNA synthesis, to kill all still-dividing cells at a critical time in the cultures differentiation; and (*iii*) use of unprecedented high concentrations of insulin and glucose in the medium.

MATERIALS AND METHODS

Cell Suspension. The sources of neurons were fetal mice (NIH strain), fetal rats (Wistar Lewis–White strain), or fetal hamsters (263-K strain). Embryos were taken from mice at the 11th day of gestation, from rats on the 11th day of gestation, or from hamsters on the 9th day of gestation. Litters of all three species usually contained 8–10 fetuses. Although all species of rodents tested yielded identical results, we report here the preparation and growth of cultures from fetal mice.

The pregnant mice were killed by decapitation and the embryos were immediately removed by aseptic surgical procedures. Pregnant hamsters or rats were anesthetized for removal of the embryos. The mouse embryos, each about 6 mm long, were placed in a sterile petri dish and immediately the whole cephalic region was amputated with iridectomy scissors and placed into a 50 ml centrifuge tube with 10 ml of medium no. 1 (see below) previously warmed to 37°C. The tissue was then gently dissociated mechanically by passage through an 18-gauge needle into and out of a 20-ml syringe five times; thereafter, the needle was replaced successively by gauges 19, 20, 21, and 22, and the syringe was filled and emptied five times through each needle size.

Culture Dishes. A sterile coverslip was set into each of 50 35-mm Falcon tissue culture plastic petri dishes (in these cultures there is no need for coating the glass coverslips with collagen or gelatin). Three milliliters of medium no. 1 was added to each culture dish, after which the dishes were warmed by placing them in an incubator for 1 hr at 37°C prior to the preparation of the suspensions of embryo tissue.

Ten drops (0.2 ml) of cell suspension were then carefully added into each dish directly within the incubator in order that the dishes need not be moved after the addition of the cell suspension. These cells then settled slowly upon the coverslip.

The cells were incubated in a humified atmosphere of 10% CO_2/90% air at 37°C without changing the medium during the first 6 days, during which the culture became confluent. The medium was replaced on day 7 by medium no. 2 which was then used as a maintenance medium, and changed every 6 days. After day 13 in culture the arabinosylcytosine could be omitted from medium no. 2.

Growth and Maintenance Media. Medium no. 1 contained the following: 80% Dulbecco's modified medium (Microbiological Associates, Bethesda, MD), 10% fetal bovine serum (Microbiological Associates), 10% horse serum (Flow Laboratories, McLean, VA) inactivated at 56°C for 30 min, dextrose at 10 g/liter, crystalline insulin at 80 units/liter (Sigma), $NaHCO_3$ at 1.5 g/liter, penicillin at 5000 units/ml, streptomycin at 5000 μg/ml (Flow), and 200 mM L-glutamine at 10 ml/liter. Medium no. 2 was as above but without the fetal bovine serum, with the horse serum at 20%, and with arabinosylcytosine at 10 mg/liter (Sigma).

Silver Staining of Neurons. Cells on coverslips were fixed *in situ* for 1 hr with 4% formaldehyde in phosphate-buffered saline, washed in phosphate-buffered saline, and then placed for 15 sec in cold acetone (−20°C). Bodian or Sevier–Munger methods of silver impregnation for neurons and neurofibers were then used (9).

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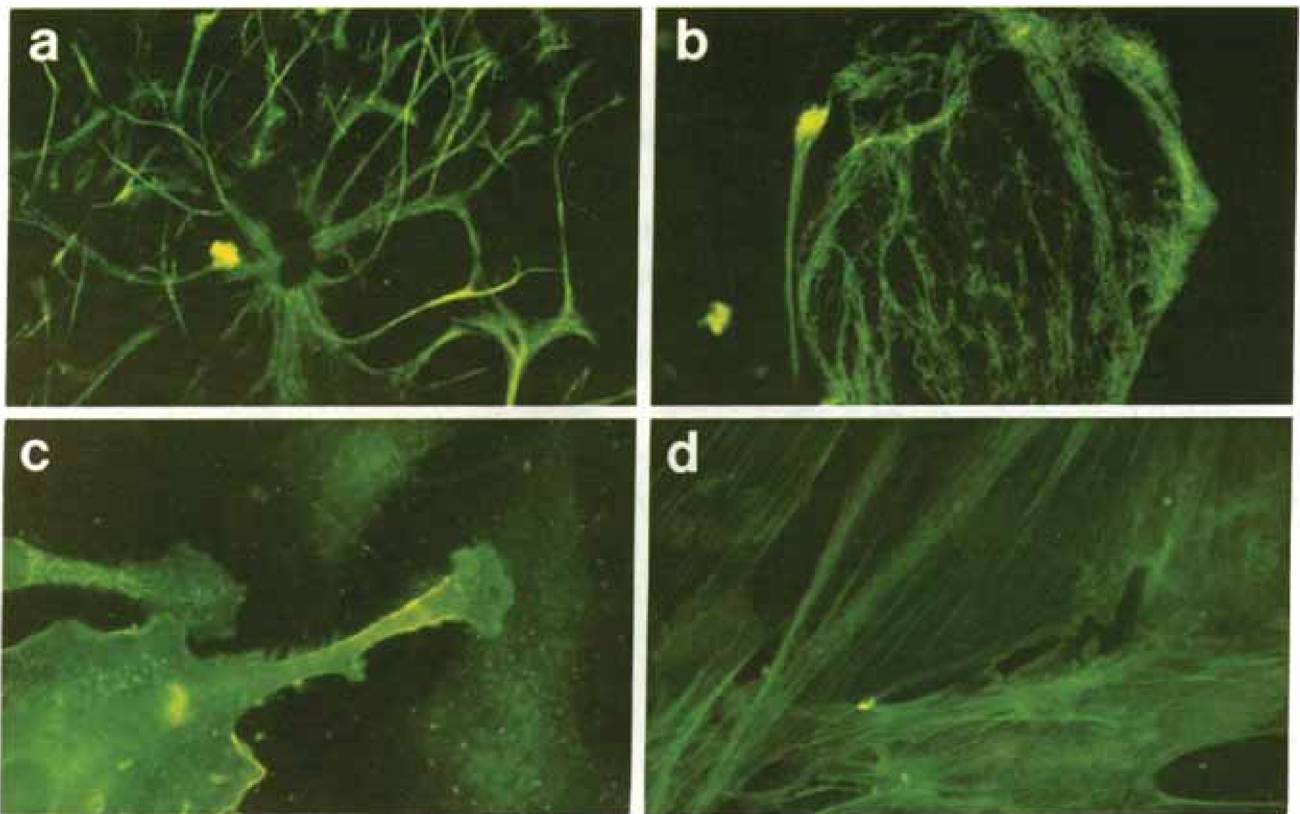

FIG. 1. Neurons connected by neurites after 20 days in culture. (*a*) Living neurons lying upon a supporting layer of glial and fibroblastic cells. (Phase contrast; ×100.) (*b*) A complex network of neurites connecting neurons in culture. (Phase contrast; ×100.) (*c*) Neuron culture, showing dense neuron clusters and their intercommunicating neurites. (Bodian silver stain; ×25). (*d*) Bundle of axons, some of them making a 90° turn to follow another group of axons. (Bodian silver stain; ×300.)

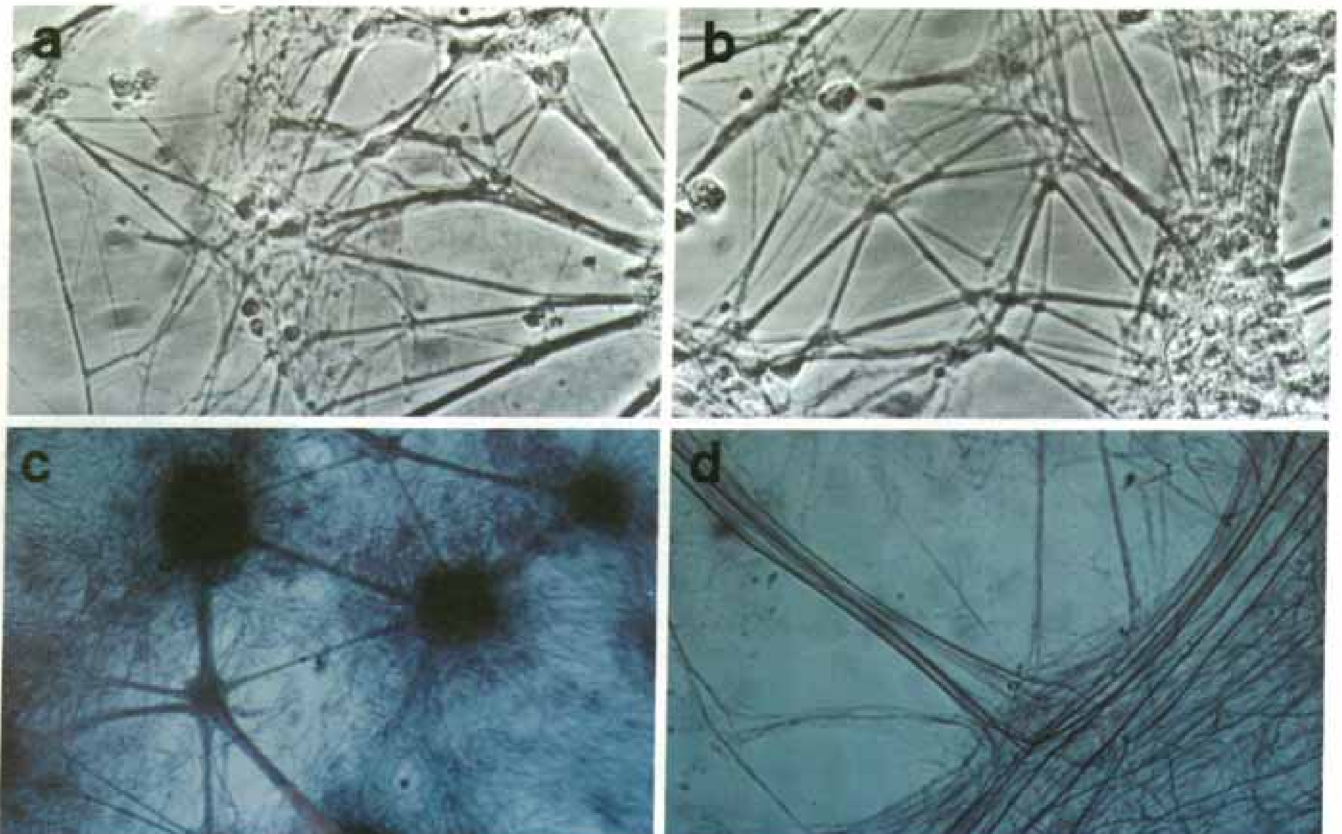

FIG. 2. Supporting cells underlying the neurons characterized by the use of specific immunological markers (×200). (*a*) Fibrillar astrocyte with its processes; the nucleus is the dark structure at the center of the cell. (Fluorescent staining, anti-glial fibrillary acidic protein antibody; 22 days in culture.) (*b*) Protoplasmic astrocyte, showing the cytoplasmic distribution of glial intermediate filaments. (Staining as in *a*; 22 days in culture.) (*c*) Two oligodendrocytes displaying protoplasmic extensions. Note the two unstained cells to the right of the oligodendrocytes. (Fluorescent staining with anti-galactocerebroside antibody; 20 days in culture.) (*d*) Group of fibroblasts, showing the cytoplasmic distribution of microfilaments. (Fluorescent staining with anti-actin antibody; 20 days in culture.)

Staining of Cells in Cell Layers Underlying Neurons. The histological character of non-neural cells was studied by using immunological markers of defined specificity as follows. Fibrillar and protoplasmic astrocytes were fluorescent-stained with rabbit antibody prepared to glial fibrillary acidic protein (obtained from Amico Bignami) (10); oligodendrocytes were identified by immunofluorescent staining with rabbit antibody to galactocerebroside prepared by the method of Raff *et al.* (11); fibroblasts were identified indirectly by two means, their lack of immunofluorescent staining with any of the former antisera and by their characteristic intracellular distribution of actin when immunofluorescently stained with rabbit anti-actin antibody made in our laboratory by the method described by Owen *et al.* (12). Indirect immunofluorescent staining was carried out on the cells growing on coverslips after they were fixed for 10 min in cold absolute methanol, rinsed in phosphate-buffered saline, and then immersed for 10 sec in cold absolute acetone. The technique used was the "sandwich" immunofluorescent test (13), applying to different coverslips each of the above mentioned specific antibodies. After staining, the cultured cells were viewed under a Zeiss photomicroscope with epi-fluorescence. Silver-stained cultures were observed in a Zeiss light photomicroscope, and living cells were studied with a Nikon phase-contrast inverted photomicroscope.

RESULTS

After 6 days in culture the appearance of most of the cells was that of a confluent fibroblast-like cell line growing among cellular debris as a result of the initial mechanical dissociation, but some special features were apparent. Many cells tended to aggregate in round clumps and it was possible to see a few short, straight processes coming out from these clumps. On day 7, nutrient medium no. 1 was replaced by maintenance medium no. 2 containing the arabinosylcytosine and increased concentration of inactivated horse serum but no fetal bovine serum. This led to marked changes in the cell population of the cultures. From the second week there was a progressive diminution of the fibroblasts with the death of many dividing cells and a spread and differentiation of other cells, particularly those located underneath and around the clumps of neurons. At this stage the neurons were easily recognized by two features: they were growing on the top of a confluent layer of cells, thus forming an upper layer of cells over the cellular substratum; and they had started to produce an increasingly complex network of long, straight processes connecting the neuron clusters or making connections with many single neurons throughout the coverslips.

By day 20 the appearance of the culture was noticeably different from that of the first and second weeks. The culture was made up largely of differentiated neurons lying upon a single layer of morphologically distinct cells (Fig. 1*a*). By the end of the third week, the upper layer of the culture consisted of many clear polyhedric cell-free areas bounded by the long, straight neuronal processes extending between the neurons which they connected (Fig. 1*b*). The supporting cells are shown in Fig. 2.

The Bodian and Sevier–Munger methods of silver impregnation of neurofibrils permit the specific identification of neural cells and their processes. With such silver staining all the background cells have a colorless appearance whereas the neurons and their processes are stained black. Most of the neurons were grouped in clusters containing 3–50 or more neurons (Fig. 1*c*). Although many neurons grew separately, they always sent out branches (Fig. 1*d*) that made contact with other neurons. A relevant feature of these long-term cultures was the running together of neurofibrils with fibrils from other neurons to form thick, straight bundles of axons which, after some distance, diverged to follow different directions (Fig. 1*d*).

Morphologically (14), some of these cells were bipolar neurons with two long branches emerging from the cell body and running in opposite directions (Fig. 3*a*). Others had a large cell body with a thick, long axon, sometimes as long as 5 mm and several hundred times the diameter of the cell body (Fig. 3*b*), ending regularly at another neuron cluster or at the edge of another neuron process. Multipolar neurons with profuse arborizations (Fig. 3*c*) also were distinguishable and some basket-like neurons resembling the Purkinje cerebellar cells were noted (Fig. 3*d*). Many other morphologically different neurons that stained specifically after silver impregnation were present in these cultures (Fig. 3 *e–h*).

Numerous dendrites emerged from the neurons but, unlike the straight thick filaments described above, these were tortuous and constituted an intricate reticulum with hundreds of thin fibers running in separate directions. Furthermore, these slender curved filaments were not apparent in the unstained culture and were seen only after silver-impregnation staining (Fig. 1*c*).

The background population of mature cells living underneath the neurons and apparently supporting them was made up mainly of astrocytes and fibroblasts with some oligodendrocytes. After immunofluorescent staining of the cultures with antibody to glial fibrillary acidic protein (10), it was possible to distinguish two kinds of astrocytes, fibrous and protoplasmic, dispersed throughout the field, many of them below or around the neurons. The fibrous astrocytes presented numerous ramified branches (Fig. 2*a*). The features that permitted them to be differentiated from neurons were the fact that fibrous astrocytes were attached directly to the glass and therefore were in the lower stratum of cells; also, their branches were curved and ramified and they did not form an interconnecting network of fibers as did the neurons. In addition, in the astrocyte the cytoplasm was extensive, and the nuclei were easy to distinguish and they failed to stain with the methods of silver impregnation used. The protoplasmic astrocytes in culture appeared to be similar to common fibroblasts; in fact, it was not possible to discriminate one from the other under light microscopy; however, after immunofluorescent staining with antibody to glial fibrillary acidic protein, all the astrocytes were brightly stained (Fig. 2*b*) whereas the rest of the cells, including fibroblasts and neurons, remained dark.

The oligodendrocytes were localized by using an antibody directed against galactocerebroside, a glycolipid hapten from myelin (11). They had a characteristic appearance with some pseudopods emerging from the cytoplasm and spreading to form a round sheet on the glass surface (Fig. 2*c*). These cells also attached to the glass and clustered near the neurons.

Most of the remaining cells were fibroblasts which persisted unstained throughout all the above staining techniques; when these cells were treated with anti-actin antibody in the immunofluorescent test, they revealed the peculiar distribution of microfilaments in fibroblasts described in detail by Lazarides and Weber (15) (Fig. 2*d*).

It was difficult to establish the exact proportions of each histologic type of cell in these cultures because they varied slightly from one coverslip to another, although our experience was that by the fourth week in culture about 80% of cells were fully differentiated neurons lying upon a layer of mixed cell population of 10% astrocytes, either fibrous or protoplasmic, 2% oligodendrocytes, and 8% fibroblast-like cells.

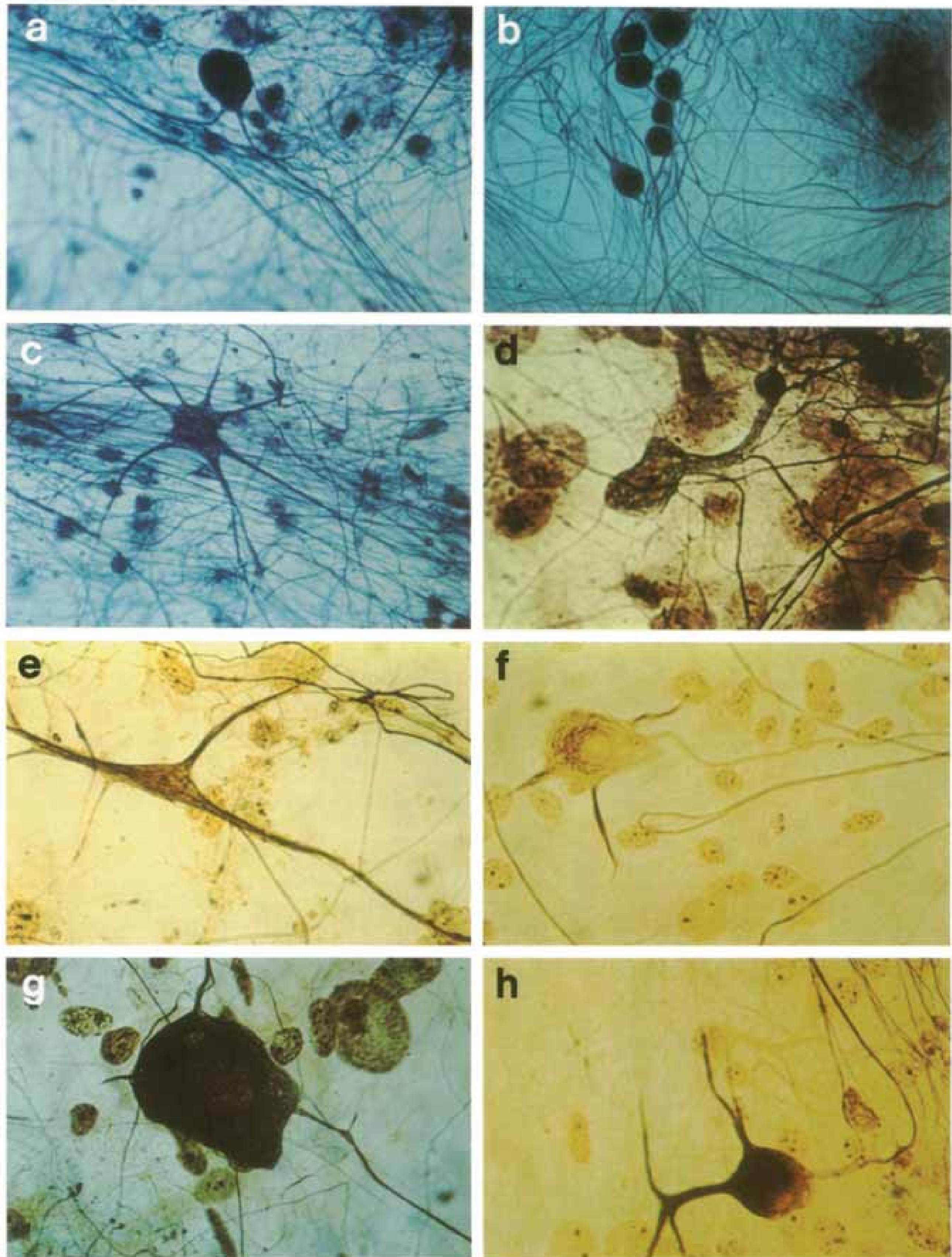

FIG. 3. Various cell types of neurons after 20 days in culture stained with the Bodian (B) or Sevier–Munger (S-M) silver impregnation method (×200). (*a*) Bipolar neuron (B). (*b*) Monopolar neurons (B). (*c*) Multipolar neuron (B). (*d*) Basket-like neuron with many protruding neurites (S-M). (*e*) Triangular neuron (S-M). (*f*) Neuron, showing the cytoplasmic arrangement of neurofibrils and three short thick dendrites and long axon (S-M). (*g*) Giant neuron (B). (*h*) Neuron with two thick, short processes and a thin, long extension (S-M). The shape of these neurons resembles sensory ganglionic neurons (*a*), cells from the trigeminal nucleus (*b*), motor neurons (*c*), Purkinje cell (*d*), pyramidal cell (*e*), pyriform neuron (*f*), Dieter's neuron (*g*), and spinal sensory neuron (*h*).

DISCUSSION

We dissociated the cells mechanically from the whole cephalic region without using proteolytic enzymes. At the time chosen for its use, the fetus had a gelatinous consistency and therefore the physical dispersion of the tissue occurs without a considerable loss of cells. Selective dissection of the whole or portions of the brain is difficult and time consuming and has offered little advantage for our studies. The initial cellular suspension is a mixture of many neural and non-neural cells. In order that the cells may follow as well as possible the natural process of maturation, we permit them initially to rearrange and form confluent layers. After 1 week the culture is overcrowded, at which time the addition of arabinosylcytosine will kill selectively the dividing cells. This antimetabolite is superior to fluorodeoxyuridine in neuron cultures (16), had no apparent toxic effect on the neuron culture even after a long exposure, and has no toxic effect upon the nervous system when used in patients (fluorodeoxyuridine has cerebellar toxicity). At the time of addition of the arabinosylcytosine the neuroblasts had started cell maturation and no longer were undergoing mitosis. The great decrease of rapidly dividing cells that occurs permits the full unencumbered maturation of neurons and of some glial cells that had already ceased to divide.

The addition to the medium of large amounts of glucose and insulin to promote its utilization has critical bearing on the maturation and long-term survival of neurons. Our results support the idea that insulin may be a growth stimulant factor for central neurons. The inclusion of small amounts of antibiotics in the medium has no visible adverse effect upon the neurons.

The neurons grow to form a network of processes on top of a population of supporting astrocytes, fibroblasts, and oligodendrocytes. Many neurons appear to grow upon fibroblasts only, with no astrocytes among them. This casts doubt on the need for astrocytes to support the growth of neurons *in vitro* (3). In our cultures there are over 4 times the number of neurons as supporting cells.

This simple method can provide a large population of central neurons living *in vitro* for immunological, microbiological, and biochemical studies.

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