Proc. Nat. Acad. Sci. USA
Vol. 71, No. 9, pp. 3575–3579, September 1974

Establishment of a Clone of Mouse Hypothalamic Neurosecretory Cells Synthesizing Neurophysin and Vasopressin

(fetal nerve cells/simian virus 40/ultrastructure/isoelectric focusing/immunoassays)

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Communicated by André Lwoff, July 1, 1974

ABSTRACT Hypothalamic cells taken from 14-day-old mouse embryos were cultured for 6 days and transformed with simian virus 40. After cloning, a homogeneous cell population was obtained. Its morphological, ultrastructural, biochemical, and immunochemical properties were studied. These cells possess ultrastructural features of primitive neurosecretory cells. They synthesize ^{35}S-labeled protein components that have the molecular weight and isoelectric focusing behavior of, and display the same immunoreactivity as, neurophysin. In addition, a ^{35}S-labeled peptidic fraction with a molecular weight close to 1000 is synthesized and is radioimmunologically indistinguishable from vasopressin. Immunochemical staining shows that both neurophysin and vasopressin are localized in the cytoplasm. These observations strongly suggest that a clone of mouse hypothalamic neurosecretory cells has been obtained with the synthesizing capacities of secretory neurons of the magnocellular hypothalamic nuclei.

Mouse fetal hypothalamus offers a valuable model for studying the pattern of biochemical differentiation characteristic of brain maturation and the regulation of the synthesis of specific hypothalamic hormones elaborated into secretory neurones. The hypothalamus undergoes a slow maturation during both fetal and post-natal life and elaborates several neurohormones and mediators which can be used as markers of cell differentiation. Other studies from this laboratory allowed us to define culture conditions for fetal mouse hypothalamic cells of various ages and to describe their pattern of differentiation in culture, as observed by phase contrast§ and by electron¶ microscopy.

This paper reports a hypothalamic cell line obtained after transformation by simian virus 40 (SV 40) of dissociated and cultured hypothalamic cells from 14 day-old mouse fetus. After cloning, a homogeneous cell population has been isolated and maintained in culture for 18 months (38 passages). These cells possess ultrastructural features of highly functional secretory cells and of primitive nerve cells. This suggests that they could elaborate the secretory material characteristic of magnocellular hypothalamic neurons: neurohypophysial nonapeptide hormones and their carrier proteins, the neurophysins (1). Proteins that share biochemical and immunochemical properties with neurophysin and that are associated with vasopressin were characterized in cellular extracts, by means of gel filtration, isoelectric focusing, and radioimmunoassay, as well as in whole cells by means of immunochemical staining.

Abbreviations: PBS, phosphate-buffered saline; AR IgG-HRP, anti-rabbit immunoglobulin bound to horse radish peroxidase; RIA, radioimmunoassay; SV 40, simian virus 40.

§ Ph. Benda, F. De Vitry, and A. Tixier-Vidal, "Patterns of organization in dissociated cell cultures of foetal mouse hypothalamus. I. Phase contrast microscopy," submitted for publication.

¶ A. Tixier-Vidal, Ph. Benda, F. De Vitry, and R. Picart, "Patterns of organization in dissociated cell cultures of foetal mouse hypothalamus. II. Ultrastructural study," submitted for publication.

MATERIAL AND METHODS

Cell Cultures. Mouse hypothalami were dissected out of 14 day-old mouse embryos (A/J strain). After removal of the meninges, they were washed thoroughly in phosphate-buffered saline (PBS), then cut into small fragments. The tissue fragments were dispersed into single cells by flushing 10 to 15 times through a fine pasteur pipette. The resulting cell suspension was distributed into 30-ml Falcon plastic tissue culture bottles (approximately three hypothalami, 3×10^6 cells, per bottle) containing 2 ml of the following medium: Ham F 10 plus 15% heat-inactivated horse serum, 2.5% fetal-calf serum, 1.5% of 200 mM L-glutamine, penicillin (50 IU/ml) and streptomycin (50 μg/ml). The bottles were incubated at 37° at saturation humidity in a 10% CO_2–90% air atmosphere.

Continuous cell lines were grown on the same medium, which was changed every 2–4 days by removing half of the medium and replacing an equal volume of fresh medium. Subcultures were initiated at intervals varying from 2 months to 1 week. Clonal cell lines were isolated by single cell plating in Falcon micro-well trays, and selection of colonies was done according to morphological criteria.

SV 40 Virus Infection. Before infection, the primary cultures were grown for 6 days. The cultures were washed once with serum-free medium and covered with 1 ml of SV 40 suspension containing 7×10^7 plaque-forming units/ml (kindly provided by Dr. M. Girard and F. Suarez from the Cancer Institute of Villejuif). After adsorption for 30 min at 37°, the layers were covered with normal culture medium and incubated at 37°. Culture bottles of the same series were untreated and kept for 2 months as control.

Chromosome Analysis. Chromosome spreads were prepared from logarithmic phase cultures after 4-hr treatment with 1 μM colchicine. Washed cells were swollen for 20 min at 37° in 1% sodium isocitrate and ethanol (1:3) and squashed.

Ultrastructural Studies. Cells were fixed *in situ* as previously described¶ and embedded *in situ* in Epon (2). Small areas of the cultures were selected, cut out, and mounted on an Araldite

block. Pale gray sections were examined after staining with uranyl acetate and lead citrate.

Isolation of Labeled Neurophysin-Like Proteins and Polypeptides. Cells taken at the end of the logarithmic growth phase were grown in 70 ml of culture medium containing 8 μCi/ml of L-[^{35}S] cysteine (65 mCi/mmole, CEA Saclay) for 48 hr. They were washed with PBS, scraped, centrifuged at $500 \times g$ and resuspended in cold 0.1 M HCl. After disruption by sonication, the homogenate was allowed to stand 18 hr at 4°. The insoluble material was then removed by centrifugation. Tris (10 mg/ml) was added to the supernatant and the pH was adjusted to 7.0. The resulting precipitate was discarded after centrifugation and the supernatant was subjected to gel filtration on Sephadex G-75 in 0.1 M HCOOH after the pH had been adjusted to 2.7. The radioactive effluent fractions corresponding to elution volumes of the neurophysin and hormones were pooled separately and lyophilized.

Isoelectric Focusing. Isoelectric focusing on the protein recovered from the G-75 column was carried out as described previously (3, 4) using an LKB (110-ml) column and LKB ampholine (40% w/v; LKB Produkter, Uppsala, Sweden). The pH gradient range was from 4 to 5.2.

Two different types of experiments were run. In one case, isoelectric focusing was performed in the presence of additional radioactive bovine neurophysins (3) as internal standard. In the other case, when focusing was done in order to separate the different labeled protein species synthesized in the cell cultures, no crude bovine neurophysins were added. The fractions corresponding to each peak were pooled, freed from ampholine and sucrose, then lyophilized and assayed for neurophysin II (see below).

Radioimmunoassay. Neurophysin was measured by radioimmunoassay (RIA) (5). The assay was set up with bovine neurophysin II as standard and tracer, and with rabbit antiserum to neurophysin II. Antibody was induced in rabbits with pure bovine neurophysin II prepared according to Breslow (4). This material contained no neurophysin I contaminant on polyacrylamide gel electrophoresis. This antiserum was specific for bovine neurophysin II and cross-reacted very poorly with bovine neurophysin I. This assay therefore measured almost exclusively bovine neurophysin II and allowed measurement of 100 pg of neurophysin II.

Assays were performed on the material with a molecular weight of 10,000 obtained after Sephadex G-75 purification of cell extracts, and on material corresponding to the various peaks of the electrofocusing preparation made in the absence of bovine neurophysins.

Vasopressin was measured by RIA (6) using an antiserum obtained in rabbits immunized with vasopressin coupled to thyroglobulin (7). This method allowed measurement of 5 pg of arginine vasopressin. An RIA was developed for oxytocin. The methods used to obtain oxytocin antisera, for the iodination procedure, and the conditions of the assay were the same as for vasopressin. The sensitivity of this method was 150 pg. Assays were performed on the material having a molecular weight of 1000 obtained by Sephadex G-75 chromatography of cell extracts.

Immunocytochemical Methods. (a) For detection of SV 40 T antigen, immunofluorescence was carried out in collaboration with C. de Vaux St Cyr (Cancer Institute of Villejuif). Cells were grown on 15-mm cover glasses in Leyton tubes for 24 hr, washed with PBS, air-dried, and fixed with methanol–ether (2:1) for 10 min at 4°. The cells were incubated at room temperature with anti-T antigen hamster serum or normal hamster serum for 30 min, then washed with PBS buffer. Fluorescein-labeled rabbit antiserum to hamster globulin (Pasteur Institute) was then added to the cells for 30 min. After washing with PBS the cells were mounted in phosphate-buffered glycerol (pH 7.5) and examined.

The serum used for the detection of SV 40 T antigen was obtained from hamsters bearing tumors after injection of SV 40 transformed cells. As controls, pre-immune hamster serum preabsorbed with hamster cells and calf serum, or fluorescein conjugate alone were used.

(b) Neurophysins, vasopressin, and oxytocin were sought by means of the peroxidase-labeled antibody technique and a 1:4–1:10 dilution of the same antiserum as was used for radioimmunoassay. HT9-C7 cells grown in plastic dishes were treated according to Tougard et al. (8). The specificity of the reaction was tested by using normal rabbit serum or specific antisera previously absorbed by their homologous antigens as described in Table 1. Control experiments were performed on mouse neuroblastoma cells (S20) and mouse embryo SV 40 transformed fibroblasts (SV 3T3).

RESULTS

Morphological Features. One month after addition of the virus, foci of dividing cells appeared. These cells, called HT9, were collected and serially transferred (three times) for $2^1/_2$

TABLE 1. *Immunochemical staining of HT9-C7 cells*

Cells	Specific antibody 1:10	Nature and amount of absorbing antigen per ml*	Intensity of staining†
Ht9-C7	Anti-neurophysin II	0	++++
		Crude neurophysin (200 μg)	+
		Neurophysin I (100 μg)	+
Ht9-C7	Anti-Lys-vasopressin	0	+++
		Arg-vasopressin (6 IU)	±
Ht9-C7	Anti-oxytocin	0	±
		Oxytocin	±
S20	Anti-neurophysin II	0	±
SV 3T3	Anti-neurophysin II	0	−
Ht9-C7	Normal serum + AR IgG-HRP		−
	AR IgG-HRP		−
S20	Normal serum + AR IgG-HRP		−
	AR IgG-HRP		−
SV 3T3	Normal serum + AR IgG-HRP		−
	AR IgG-HRP		−

AR IgG-HRP is anti-rabbit immunoglobulin bound to horse radish peroxidase.

* Antibody absorption by antigens was performed for 3 days at 4°.

† Number of +s is proportional to the intensity of the staining; −, no reaction.

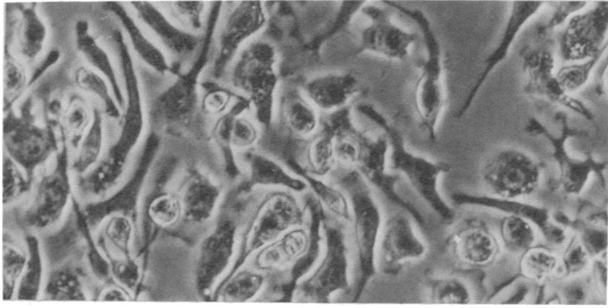

FIG. 1. HT9 C7 cells; phase contrast microscope; ×240.

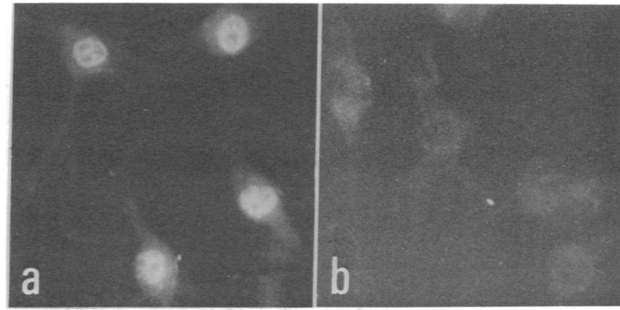

FIG. 2. HT9 C7 cells; (*a*) localization of T antigen within the nucleus; (*b*) control cells; ×340

months. Electron microscope study performed after three and five transfers revealed a heterogeneous cell population composed of fibroblasts and cells characterized by a prominent Golgi zone with secretory material and dense bodies. After the third passage, 10 different clones were isolated. Progressive changes in morphology of cells within the clones appeared after successive passages. Clone C7 was considered to be one of the most differentiated, according to morphological criteria, such as length of cytoplasmic processes and tendency to reaggregate. It possessed a doubling time of about 24 hr and a mean chromosome number of 106 ± 5 instead of 40–120 in the original heterogeneous strain. When the cultures were sparsely populated, cells had a multipolar shape, with one or two long processes (Fig. 1) ended by a refractile "bouton." When they were densely populated, the cells became rounded and tended to clump together and float.

An ultrastructural examination was performed periodically beginning 4 months after the clone had been isolated. The examinations gave consistent results. The cells possessed morphological features typical of an intense secretory activity. The very large Golgi zone contained abundant microvesicles, coated vesicles, and several stacks of four to six saccules. Dense material was often found within vesicles, rounded secretory granules (100 nm), and elongated cisternae. In addition, numerous large dense bodies or secretory granules of irregular shape and size were often seen within the perikaryon and the cytoplasmic processes. Many free ribosomes and polysomes, as well as some linear rough endoplasmic reticulum cisternae, and a few scattered microtubules and filaments occupied other parts of the cytoplasm. The long cytoplasmic processes generally contained ribosomes and mitochondria and, in a few cases, narrow strips of secretory material, elongated smooth cisternae, and a few microtubules. They ended by a growth cone with filopodia, a typical embryonic structure of growing neurones. No true axon or axon terminal was found. Within the nucleus, the karyoplasm appeared light and chromatin formed a marginal layer and scattered clusters.

Immunofluorescent Staining for T Antigen. In nearly 100% of the cells, T antigen was clearly localized within the nucleus as revealed by the immunofluorescence technique. In the controls, only a faint yellow fluorescence was seen in the cytoplasm (Fig. 2).

Immunocytochemical Localization of Neurophysin, Vasopressin and Oxytocin. Results are summarized in Table 1. The C7 cells alone presented a strong positive reaction with antisera against bovine neurophysin II. Previous absorptions of this antisera with either neurophysin I or crude bovine neurophysin (I and II) confirmed the specificity of the immunochemical staining and suggested that the cells may contain neurophysin-like proteins. They also gave a positive reaction with an anti-vasopressin. Negative results were obtained with the anti-oxytocin.

Isolation of Labeled Neurophysin-Like Proteins and Polypeptides. Neurophysin-like proteins and hormones, labeled with [^{35}S] cysteine were found in 0.1 M HCl cell extracts. Approximately 50% of the total protein extract was soluble in 0.1 M HCl. Precipitation at pH 7 (9) eliminated 50% of the 0.1 M HCl, soluble proteins. Neurophysin-like proteins, together with proteins of a molecular weight of 10,000 (4), were then separated from high-molecular-weight proteins and smaller peptides by gel filtration on Sephadex G-75. They corresponded roughly to 10% of the total protein in the cell extract.

Characterization of Labeled Neurophysin-Like Proteins. The radioactive material with a molecular weight of 10,000 separated by gel filtration was subjected to isoelectric focusing. Fig. 3*a* shows the elution pattern obtained when electrofocusing was carried out in the presence of added crude bovine neurophysins. The pattern of the eluted radioactive material exhibits four main peaks corresponding to different molecular species. Three of them, A, B, and C, appeared to behave like bovine neurophysin I, II, and III with an apparent pI of 4.28, 4.67, and 4.35, respectively. In addition a significant amount of radioactive material with an apparent pI of 4.54 was always found (peak D). A very similar pattern was found in all the three separate experiments run on different cell cultures.

Radioimmunological Assays. Neurophysin was found in all of the three different extracts assayed. Fig. 4 illustrates a dilution experiment of the assayed material. It clearly shows that inhibition curves obtained by adding increasing amounts of immunoreactive material were very similar to standard curves, although slight differences appeared. The amounts measured with the bovine neurophysin II standard are indicated in Table 2.

When neurophysin was assayed in different fractions obtained from electrofocusing (Fig. 3*b*), it was found almost exclusively in peak A (154 ng); small amounts were measured in peak D (20.5 ng) and peak B (7.7 ng). Relative values were respectively 84.2%, 11.2%, and 4.6% of the total amount eluted (peak C was not tested).

In four experiments vasopressin was found in various amounts (Table 2). A complete parallelism was found between the standard curve and the immunoreactive material. Measurement of oxytocin was performed in the same extracts. No immunoreactive material was found, although the volume of

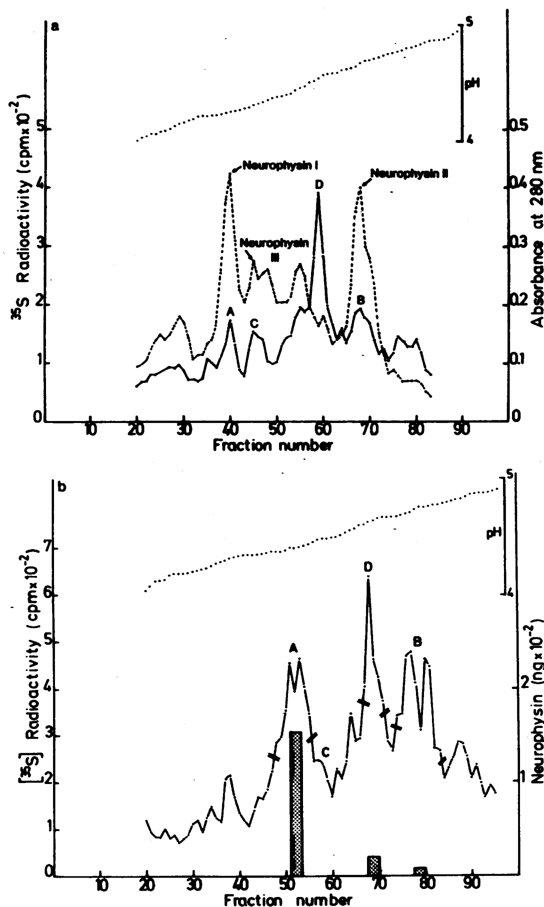

FIG. 3. Isoelectric focusing of radioactive protein material (molecular weight 10,000) obtained after Sephadex G-75 filtration. Experiments were run at 6° in an 110-ml LKB column with a mixture of ampholines pH 4–5.2 and an applied power of 1.5–2 W. After 70 hr the contents of the column was recovered in a fraction collector. The pH (dotted line) was measured on each 1-ml fraction. (*a*) Experiment carried out in the presence of crude bovine neurophysins (24 mg). The absorbance at 280 nm (broken line) and the ^{35}S radioactivity (solid line) were measured on each fraction. Radioactivity was determined on 100-μl aliquots. (*b*) Experiment carried out without added bovine neurophysin. The radioactivity of each fraction was measured on a 20-μl aliquot. The double dashes indicate the fractions of the peaks that were pooled and subjected to RIA. The shaded bars indicate the amount of immunoreactive material found in the assayed fractions with antibodies against bovine neurophysin II.

aliquots assayed was 30 to 40 times larger than those used for the arginine-vasopressin assay. These negative results cannot, therefore, be explained on the basis of difference in sensitivity between arginine–vasopressin and oxytocin radioimmunoassay.

DISCUSSION

A continuous cell line has been obtained from 14-day-old mouse fetal hypothalamic cells by infection with simian virus 40. The presence of T antigen within the nucleus, as revealed by immunochemical staining, enabled the identification of SV 40 genetic information in the HT9-C7 cell line. This strongly suggests that those cells were transformed by the SV 40. When similar cultures of 14-day-old fetus were kept for 2 months without infection with SV 40, no foci of dividing cells were observed.

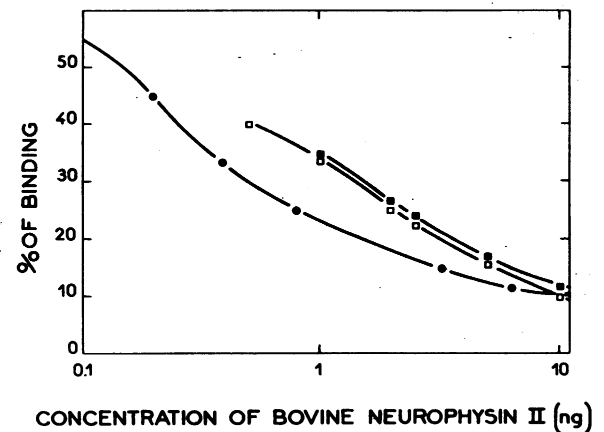

FIG. 4. Comparison of cross-reactivity of bovine neurophysin II (●) and two purified cell extracts (□, experiment 2; ■, experiment 4) as studied by RIA. The preparations were dissolved in barbital buffer and increasing volumes from 5 to 100 μl were assayed.

The HT9 clonal cell line possesses ultrastructural characteristics of secretory cells, as indicated by the considerable extension of the Golgi zone and the accumulation of secretory material and of dense bodies. These cells do not present the typical features of mature neurons, since they are devoid of axons and axon terminals. They nevertheless display features of primitive nerve cells, such as the distribution of chromatin within the nucleus, the tendency to form long and narrow cytoplasmic processes which end in growth cones and sometimes contain a few parallel microtubules, elongated smooth cisternae, and strips of secretory material. In contrast, secretory neurons accomplish *in vitro* a complete morphological differentiation when 14-day-old foetal cells are grown in the absence of SV 40 for one to two months¶.

After long term incubation of cells with [^{35}S]cysteine, the presence in cell extracts of ^{35}S-labeled protein material, which is soluble in 0.1 M HCl and which can be purified on Sephadex G-75, indicates unambiguously the synthesis by these cells of protein components, whose apparent molecular weight and isoelectric focusing behavior are characteristic of neurophysins. In addition, these protein components are recognized by an anti-bovine neurophysin II. Immunochemical staining allows the specific localization of neurophysins within the cells and their processes. The fact that an anti-bovine neurophysin II was used for our immunochemical studies does not allow us to stipulate that neurophysin II is produced by C7 cells. First of all, the biochemical and immunological properties of mouse neurophysins are still unknown. A cross-reaction has been shown between anti-bovine neurophysin sera and rat or guinea-pig neurophysins (10, 11). The similarity of the dilution curves obtained with the extracted material and with the bovine neurophysin II standard indicates that these two materials possess common antigenic determinants. Nevertheless, the apparent higher content of radioimmunoassayable neurophysin II within peak A, which presents the isoelectric focusing behavior of bovine neurophysin I, is actually difficult to explain.

Besides neurophysin-like proteins, C7 cells also synthesize an ^{35}S-labeled peptidic material of molecular weight close to 1000 which is radioimmunologically indistinguishable from arginine–vasopressin. The same conclusion can be drawn from the immunochemical staining. On the other hand,

TABLE 2. *Recovery of protein, neurophysin, and hormones from cells extracts*

	Experiment number				
	1	2	3	4	5
Number of cells × 10^{-7}	10	35	6	6	50
Protein extracted* (mg)	30	70	20	22.8	110
Neurophysin† (ng)	ND	494	ND	50	182
Vasopressin† (pg)	ND	1400	150	210	ND
Oxytocin†	S	S	S	S	S

S, below sensitivity of the assay; ND, not done.

* Measured according to Lowry *et al.* (12).

† Measured by RIA.

negative results were obtained with an antioxytocin in both radioimmunoassay and immunocytochemical staining.

In summary, we have presented biochemical and immunochemical evidence that HT9-C7 cells synthesize neurophysin-like proteins and vasopressin. Although further immunochemical studies on mouse neurophysins and neurophypophysial hormones are needed, HT9-C7 cells already appear to be the first clonal cell line to demonstrate *in vitro* the synthesizing capacities of neurosecretory cells of magnocellular hypothalamic nuclei. The morphological features of these cells suggest that they are derived from a precursor cell rather than from a fully differentiated secretory neuron.

We thank Dr. M. B. Vallotton for his generous gift of anti-neurophysin II sera. This work was supported by grants from the Centre National de la Recherche Scientifique (ERA 89, ATP 4913).

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