THE ULTRASTRUCTURE OF NEUROBLASTOMA GLIOMA SOMATIC CELL HYBRIDS

Expression of Neuronal Characteristics Stimulated by Dibutyryl Adenosine 3′,5′ Cyclic Monophosphate

MATHEW P. DANIELS and BERND HAMPRECHT. From the Laboratory of Biochemical Genetics, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014.
Dr. Hamprecht's present address is the Max-Planck-Institut für Biochemie, 8033 Martinsried bei München, West Germany.

A current approach to the investigation of the biochemical basis for neuronal function is the development, from neuroblastoma tumors, of clonal cell lines which grow rapidly in cell culture but which retain the ability to express one or more characteristics of differentiated neurons. These characteristics may be morphological (1–3), biochemical (1, 4), or physiological (5–8).

Recently, it has been shown that cells of some clonal lines derived by somatic cell hybridization between neuroblastoma cell lines and L cells are capable of stable expression of neuronal characteristics (9, 10) and that the cells of some of these hybrid lines can express certain neuronal characteristics to a greater degree than the neuroblastoma parent cells (9, 10). The present report deals with two clonal lines derived by somatic cell hybridization between neuroblastoma and glioma cell lines[1]. The cells of these hybrid lines are induced to differentiate by $N^6,O^{2'}$ dibutyryl adenosine 3′,5′ cyclic monophosphate (dBcAMP). The differentiated cells are neuronal in light microscope appearance[1], have membranes of high electrical activity, capable of generating action potentials and sensitive to acetylcholine[2], and have high levels of choline acetyl-transferase (CAT) activity[1]. All of these characteristics are expressed to a far higher degree in the hybrid cells than in cells of the parent neuroblastoma line. The present report is concerned with ultrastructural characteristics of these hybrid cells, with particular reference to a set of neuronal characteristics whose appearance is stimulated by dBcAMP treatment.

MATERIALS AND METHODS

Cell Culture

The clonal cell lines, designated 108CC5 and 108CC15, are somatic cell hybrids from a cross between a 6-thioguanine resistant mutant, N18TG2 (10), of mouse neuroblastoma line N18 (4), and a bromodeoxyuridine resistant mutant, C6-BU-1 (11), of rat glioma line C6 (16). The hybrid cell lines were prepared by inactivated Sendai virus-induced fusion (12) and selection in medium containing hypoxanthine, aminopterin, and thymidine (13) as will be described in detail elsewhere[1]. Cells of these clonal lines were identified as hybrids by their possession of both rat and mouse chromosomes.

[1] Hamprecht, B., T. Amano, and M. Nirenberg. Manuscript in preparation.

[2] Hamprecht, B., W. Kemper, and M. Nirenberg. Manuscript in preparation.

Cells were cultured in plastic petri dishes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) containing Dulbecco's modified Eagle's medium (DMEM; Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal bovine serum (Colorado Serum Co., Denver, Colo.) and incubated at 37°C in a water-saturated atmosphere of 90% air-10% CO_2. In addition, N18TG2 cells were cultured in the presence of 10^{-4} M 6-thioguanine and C6-BU-1 cells in the presence of 10^{-4} M bromodeoxyuridine. The hybrid cells were cultured in the presence of 10^{-4} M hypoxanthine, 10^{-6} M aminopterin, and 1.6×10^{-5} M thymidine. Cells were subcultured by gentle trypsinization (14) and plated at 5×10^4 cells/60-mm dish. The culture medium was changed every 1–3 days, depending on cell density. Some cultures were treated with 10^{-3} M $N^6,O^{2'}$ dibutyryl adenosine 3',5' cyclic monophosphate (Sodium salt, Grade II, Sigma Chemical Co., St. Louis, Mo.) beginning 2–4 days after plating. The medium of dBcAMP-treated cultures was changed every 1–2 days.

Electron Microscopy

The cultures were rinsed with cold DMEM and fixed for 1 h at 0–4°C in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3, with 5 mM $CaCl_2$. The fixed cultures were washed with buffer, postfixed in 1% OsO_4 in the same buffer, stained with 2% uranyl acetate in maleate buffer, pH 5.0 (15), dehydrated in an ethanol series, and flat embedded in Epon 812. Pieces of the embedding were mounted and sectioned in a plane horizontal to the cell monolayer. Thin sections were stained with uranyl acetate and lead citrate. Electron micrographs were taken with an Hitachi HU-12 electron microscope (Perkin-Elmer Corp., Palo Alto, Calif.) equipped with a model HK-6 tilting stage. Small pieces of rat and mouse cerebral cortex were fixed by immersion in the same fixative used for the cultures and processed in the same manner as the cultures but with 50% longer immersion at each step except postfixation.

Measurement of Electron-Transparent Vesicles

Outside diameters of electron-transparent vesicles in sections of the hybrid cells or brain cortex were measured with a metric ruler in calibrated electron micrographs taken at × 25,700 magnification and enlarged to × 157,000. The measurement error was ± 5–10%.

RESULTS

Morphology of the Parent Cell Lines

The morphology of the cells of the glial parent line, C6-BU-1 (Fig. 1 *a*), was similar to that of cells of the RG6 subclone of the original C6 line as described by Benda et al. (16) and Ryter and Benda (17). Treatment of C6-BU-1 cells with 10^{-3} M dBcAMP for 12 days caused a striking increase in the number and length of cell processes (Fig. 1 *b*). However, there was no marked change in cellular ultrastructure. The N18TG2 cells (Fig. 2 *a*) were generally rounded or polygonal in form, with one or more relatively short processes. They tended to form large clusters, one to two cells deep.

The ultrastructure of the N18TG2 cells has not been previously described, but most of the ultrastructural features have been observed in other lines of mouse neuroblastoma cells derived from the C1300 tumor (2). N18TG2 cells treated for 9 days with 10^{-3} M dBcAMP (Fig. 2 *b*) had a greater tendency to aggregate and produced longer processes than untreated cells (Fig. 2 *a*). These changes progressed no further with longer dBcAMP treatment, and the ultrastructural changes were correspondingly moderate.

Morphology of the Hybrid Cells

Although cells of hybrid line 108CC5 were larger, and grew more slowly than those of the 108CC15 line, the ultrastructure of these two lines was similar. Therefore, the present report will be restricted to a description of the 108CC5 cells, which have been more carefully examined. The untreated hybrid cells of line 108CC5 were larger than the cells of either parent line and similar in form to the N18TG2 cells (Fig. 3 *a*). A few of these cells had long, neurite-like processes, but most had short processes, as did the N18TG2 cells. The ultrastructural appearance of the untreated hybrid cells was, for the most part, similar to that of the dBcAMP-treated N18TG2 cells. The cell bodies (Fig. 4) were characterized by the presence of A type intracisternal virus-like particles, large dense-core vesicles, and mitochondria with poorly defined cristae and diffuse matrix. The processes (Fig. 5) contained both microtubules and 100 Å-thick filaments as well as mitochondria, dense-core vesicles, and polyribosomes.

Treatment of the hybrid cells with 10^{-3} M dBcAMP for 1–3 wk produced extensive changes in their light and electron microscope appearances. dBcAMP-treated hybrid cells were larger than controls and tended to form large aggregates. Many of the cells had long, thick, neurite-like processes which formed a network between the aggregates (Fig. 3 *b*). Many refractile cytoplasmic droplets, 2–10 μm in diameter, were observed adhering to cell bodies or processes, particularly in

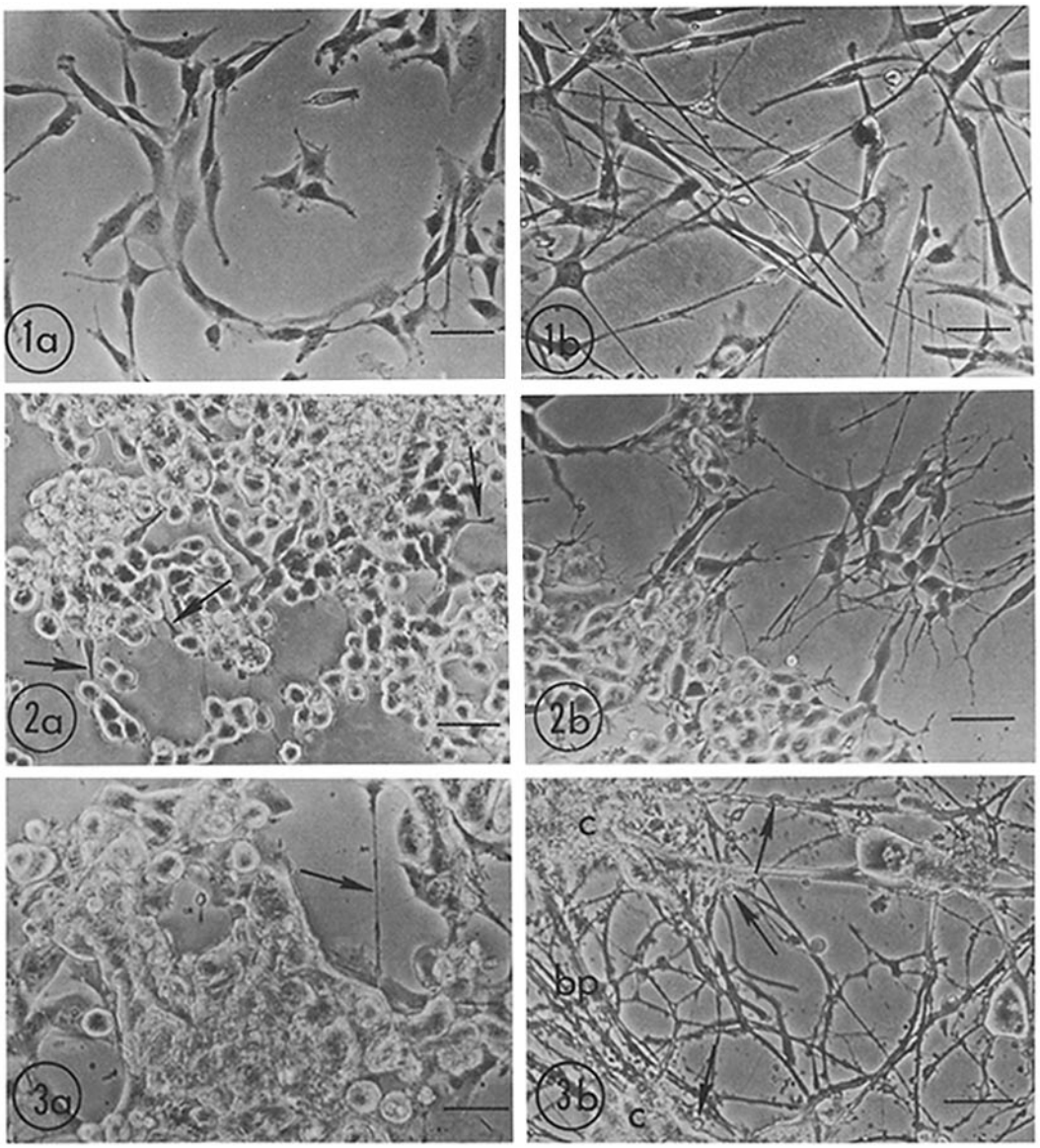

FIGURE 1 Phase-contrast micrographs of C6-BU-1 cells in culture. Fig. 1 *a*, untreated culture. Fig. 1 *b*, culture treated 12 days with dBcAMP. The cell processes are longer and narrower than in the untreated culture. × 175. Reference bars in all phase-contrast micrographs equal 50 μm.

FIGURE 2 Phase-contrast micrographs of N18TG2 cells in culture. Fig. 2 *a*, untreated culture. The cells are rounded in form and tend to form aggregates. A few short processes are visible (arrows). Fig. 2 *b*, culture treated 2 days with dBcAMP. Many of the cells have processes 2–3 times longer than the cell body. × 175.

FIGURE 3 Phase-contrast micrographs of 108CC5 cells in culture. Fig. 3 *a*, untreated culture. The cells are larger than those of the parent lines and similar in form and pattern of aggregation to N18TG2 cells. One long cell process is visible (arrow). Fig. 3 *b*, culture treated 22 days with dBcAMP. Large clusters of cells (*c*) are connected by thick bundles of cell processes (*bp*); many individual long thick processes can be seen. Droplets of cytoplasm are seen adhering to cells and their processes (arrows). × 175.

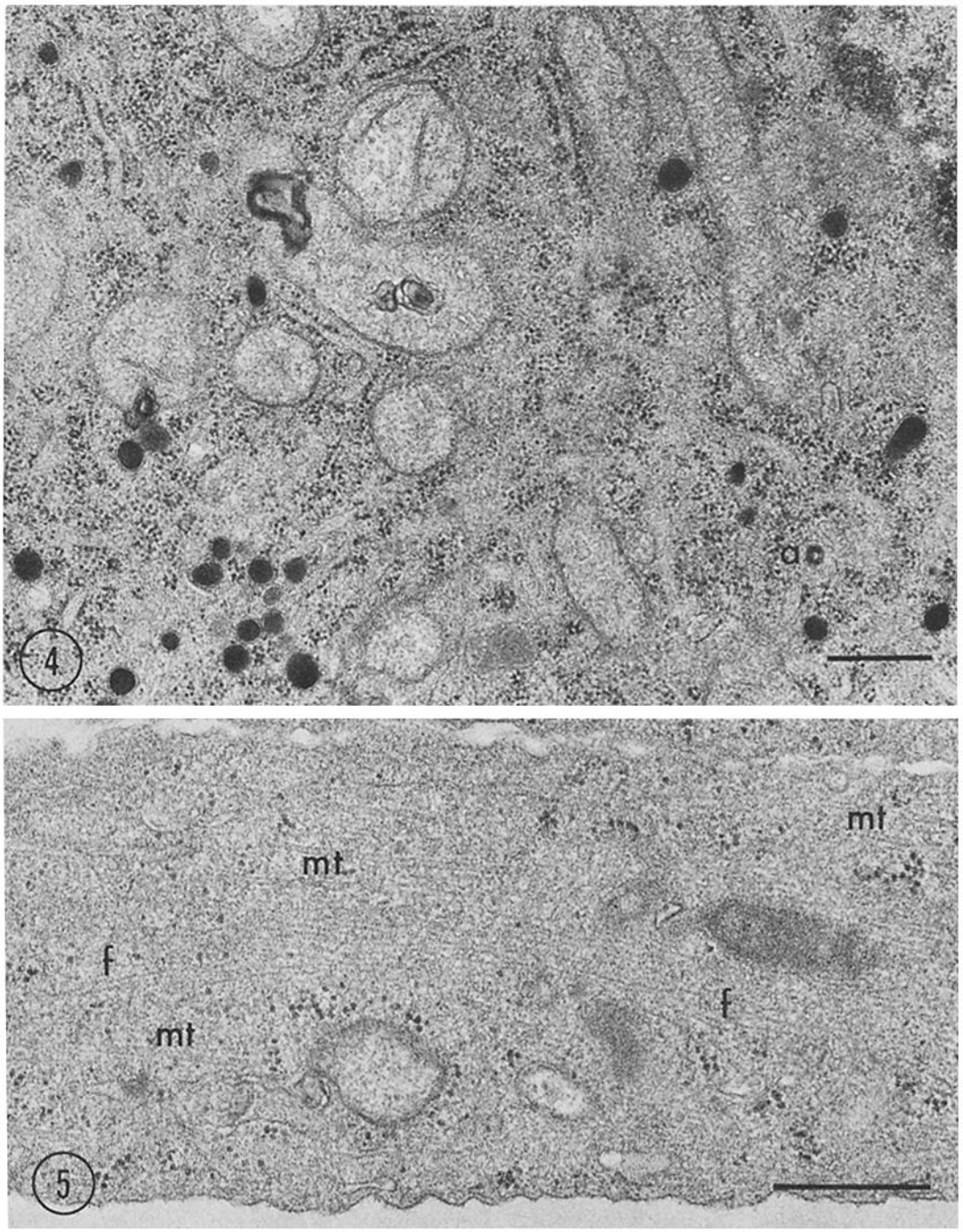

FIGURE 4 Cell body of an untreated 108CC5 cell. The cytoplasm contains many dense-core vesicles and a few A type particles (*a*). × 31,400. Reference bars in all electron-micrographs equal 0.5 μm.

FIGURE 5 Cell process of an untreated 108CC5 cell. The process has a density of microtubules (*mt*) and ribosomes (*r*) similar to that of the dBcAMP-treated N18TG2 cell processes. Numerous 100-Å thick filaments (*f*) are seen. × 46,000.

older cultures. These droplets presumably arose by budding from process endings and varicosities or, in some cases, from the periphery of cell bodies since, as described below, the organellar contents of these regions were similar. The cell processes had a characteristic ultrastructural appearance. The base of the process had more orientated mitochondria, more microtubules, and less rough endoplasmic reticulum than the peripheral region of the cell body. The main segment of the process (Fig. 6) contained a closely packed, parallel array of microtubules in addition to 100-Å thick filaments, elongated mitochondria, and cisternae of the smooth endoplasmic reticulum. Very few ribosomes were seen in the main segment. The mitochondria in the processes had a dense matrix and sharply defined cristae such as observed in axonal or dendritic mitochondria. This appearance was in contrast to that seen throughout the cytoplasm of the untreated hybrid cells (Fig. 4). The terminal expansion of the process contained many dense-core vesicles and cisternae of the smooth endoplasmic reticulum but few microtubules or 100-Å thick filaments. The most peripheral regions of the terminal expansion consisted of filopodial or web-like extensions containing only a fine filamentous network (Fig. 7). Clearly, the ultrastructure of both the main segments and the terminal expansions of the processes of the dBcAMP-treated hybrid cells closely resembled that described for dorsal root ganglion neurons (18) and sympathetic neurons (19) in dissociated tissue culture. In addition to the terminal expansions, some segments of processes were expanded and contained large numbers of organelles (Fig. 8). The contents of these expansions were variable. Some, for example, were filled with mitochondria; others with smooth endoplasmic reticulum or dense-core vesicles. As mentioned above, many refractile droplets of cytoplasm adhering to cell bodies or processes were observed in cultures of the dBcAMP-treated hybrid cells. In ultrastructure, as seen in cytoplasmic profiles of similar size, shape, and location, the contents of these droplets (Figs. 8, 9) usually resembled those of the expanded segments of processes or those of the central portions of terminal expansions. Some of the cytoplasmic droplets contained large numbers of electron-transparent vesicles, 500–700 Å in diameter, occurring singly or in clusters (Fig. 9), similar in appearance to the clear vesicles found in known cholinergic and other synaptic endings. Although most abundant in the cytoplasmic droplets, the vesicles were frequently found in expanded and terminal segments of processes and occasionally in the subterminal or the main segments of processes (Fig. 10). The vesicles could be clearly distinguished in appearance from elements of the Golgi apparatus, smooth endoplasmic reticulum, coated vesicles, and most pinocytotic vesicles by their electron transparency, clearly defined membranes, round shape, and homogeneous size distribution. The electron-transparent vesicles of 108CC5 cells had a size distribution with a peak at approximately 600 Å, 17% larger than the 500-Å peak size class for synaptic vesicles of rat or mouse brain fixed and processed similarly. The round shape of the vesicles was tested by tilting sections through an angle of 70°. This procedure revealed no significant asymmetry such as would have been observed if the circular profiles were actually cross sections of oblong cisternae. A few clusters of these vesicles were observed in the processes of 108CC5 cells not treated with dBcAMP. No more than an occasional vesicle of this appearance was seen in cells of the parent lines.

DISCUSSION

Neuron-Like Ultrastructural Characteristics of the Neuroblastoma × Glioma Hybrid Cells

Typically neuronal ultrastructural characteristics were expressed to a greater degree in cells of certain hybrid cell lines from a neuroblastoma-glioma cross than in either parent cell line. This was most clearly seen in the ultrastructure of the cell processes and endings, those of the differentiated hybrid cells appearing strikingly similar to those of normal neurons in vitro (18, 19). This enhancement of expression of differentiated neuronal morphological characteristics was correlated with the greater CAT and acetylcholinesterase activity[1] and electrical activity[2] of the hybrid cells.

The clusters of 500–700-Å diameter clear vesicles observed in the cytoplasmic droplets, process expansions, and termini, although slightly larger, were strikingly similar to those seen in cholinergic synapses. Vesicles of similar appearance have been observed in dissociated sympathetic neurons in tissue culture (20) and were reported to contain dense cores after treatment of the cells with 5-hydroxydopamine. Biochemical studies will be

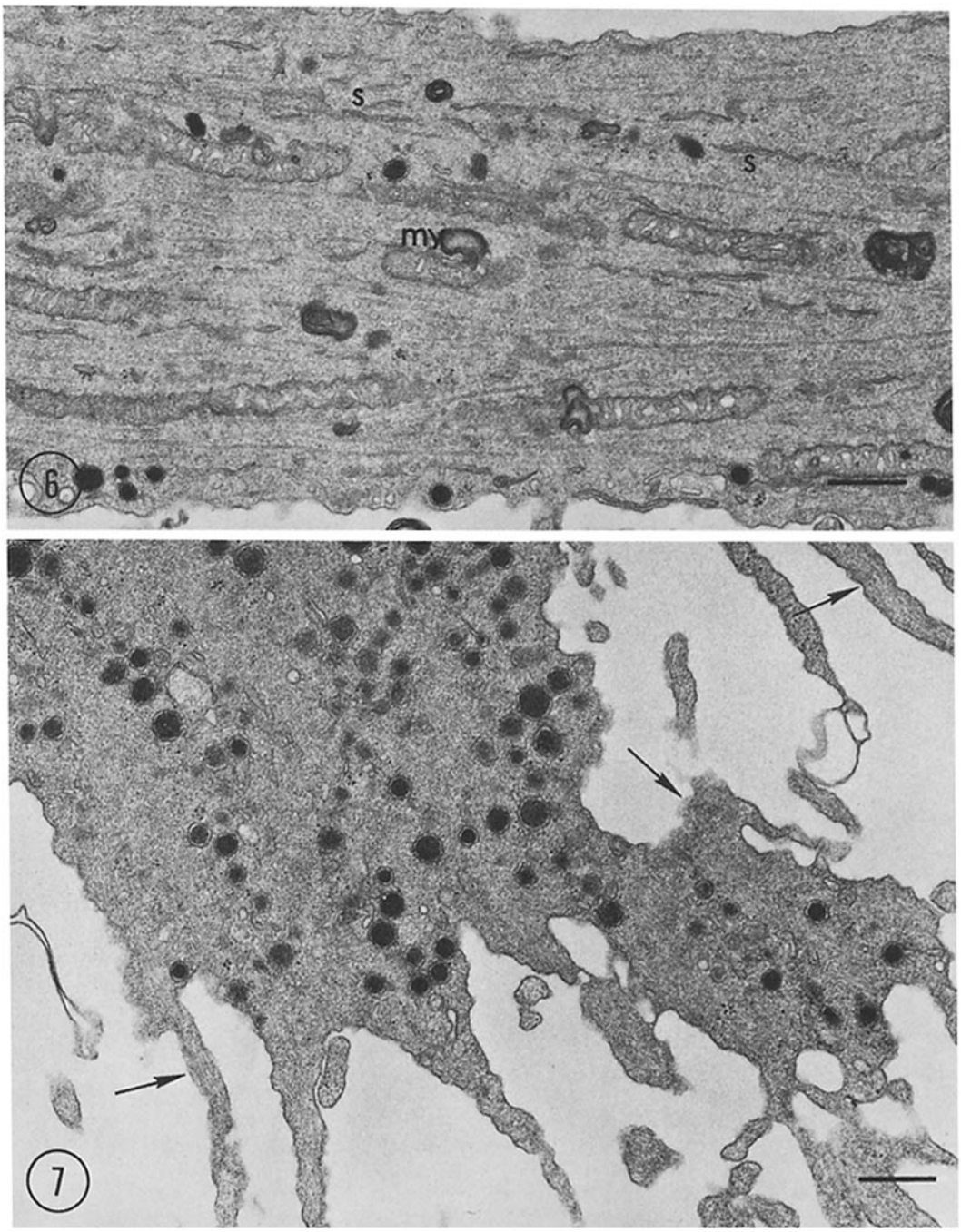

FIGURE 6 Main segment of a process of a 108CC5 cell treated 22 days with dBcAMP. The outstanding features are the parallel array of microtubules and the elongate mitochondria, some of which are associated with "myelin" whorls (*my*). Note also several dense-core vesicles and cisternae of the smooth endoplasmic reticulum (*s*). × 23,300.

FIGURE 7 Part of a large growing tip of a 108CC5 cell process after 22 days' treatment with dBcAMP. The central portion contains numerous dense-core vesicles and other vesicular elements, while the peripheral extensions contain only a fine filamentous matrix (arrows). × 23,600.

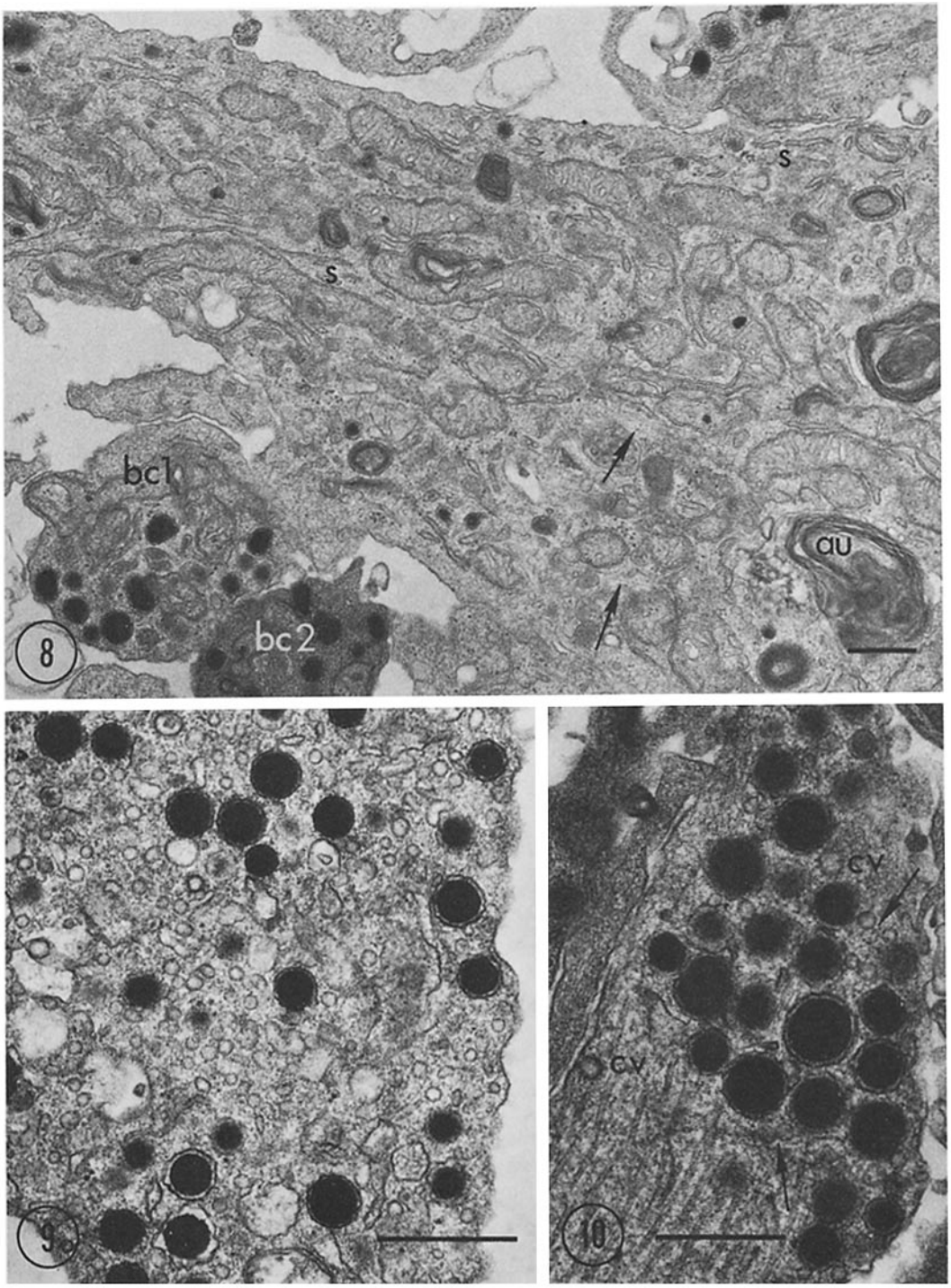

FIGURE 8 Expanded segment of a 108CC5 cell process after 22 days' treatment with dBcAMP. This segment is primarily filled with mitochondria, but smooth endoplasmic reticulum (*s*), 100-Å thick filaments (arrows), dense-core vesicles, and autophagosomes (*au*) are also present. A droplet of cytoplasm appears to be budding off of the process (bc_1), while a second droplet shows no clear point of attachment (bc_2). × 21,400.

FIGURE 9 Cytoplasmic droplet, presumably budded from a cell process of a 108CC5 cell after 22 days' treatment with dBcAMP. The droplet contains many 600-Å diameter electron-transparent vesicles in addition to dense-core vesicles. × 41,900.

FIGURE 10 Segment of a 108CC5 cell process after 22 days' treatment with dBcAMP. It contains a parallel array of microtubules and a cluster of dense-core and 600-Å diameter electron-transparent (arrows) vesicles. Notice the coated vesicles (*cv*) which are larger than the other electron-transparent vesicles. × 39,500.

required to determine if these vesicles are storage sites for some of the acetylcholine synthesized (Simpson, Deisseroth, and Nirenberg, unpublished observation) by the hybrid cells. In itself, the frequent appearance of clusters of these vesicles in the hybrid cells is of interest in that only a few isolated vesicles of similar dimensions (possibly small pinocytotic vesicles) were observed in the cells of the parent lines. It is possible that this indicates *de novo* expression of a characteristic in the hybrid cells. *De novo* expression has already been demonstrated in the appearance of CAT activity in other neuroblastoma-glioma hybrids (11) and in the synthesis of mouse specific serum albumin in hybrids between cells of rat hepatoma lines which produced albumin and mouse fibroblast lines which did not (21).

Another characteristic expressed to a greater degree in the hybrid cells than in cells of the parent lines was the presence of large dense-core vesicles. Vesicles of this appearance have been observed in other C1300 neuroblastoma cells (2, 3; Nirenberg, unpublished). Schubert et al. (2) commented on their morphological similarity to neurosecretory vesicles as well as other dense-core vesicles of unknown content and function. The dense-core vesicles of the neuroblastoma × glioma hybrid cells also resemble the chromaffin granules of the adrenal medulla (see, for example, reference 22), but the hybrid cells do not contain significant quantities of catecholamines, although they do contain the enzyme dopamine β-hydroxylase normally associated with dense-core vesicles (23).

The Response to dBcAMP

Adenosine 3′,5′ cyclic monophosphate and its analogue, dBcAMP, promote morphological differentiation in both nonneuronal neoplastic cells (24, 25) and neuroblastoma cells (26, 27). These compounds also stimulate neurite extension in cultured chick ganglia (28). In the present study, the effects of dBcAMP on the cells of the neuroblastoma parent line were noticeable, but small. The cells of the glioma parent line showed a striking response on the light microscope level, but no qualitative changes in ultrastructure were observed. The hybrid cells, however, showed a strong response to dBcAMP treatment, observable at both the light microscope and ultrastructural levels. There was a considerable difference in the time course of response to dBcAMP between the hybrid cells and the neuroblastoma cells or neurons mentioned above. Although many of the hybrid cells had extended processes after 2 days' treatment, the overall response took 1–2 wk, as opposed to the 3–48-h time courses reported by the above mentioned investigators. Since dBcAMP treatment clearly retarded proliferation of the hybrid cells[1], and since a few well-differentiated cells were observed in the untreated cultures, it is possible that in this case the effect of dBcAMP on differentiation was a result of a nontoxic growth-retarding effect of either the dBcAMP or its breakdown products, N^6-monobutyryl adenosine 3′,5′ cyclic monophosphate and butyric acid.

We thank Dr. Henry de F. Webster, Dr. Milton W. Brightman, and Dr. Marshall Nirenberg for their criticism of the manuscript, and Mrs. Alice Ling for her skillful technical assistance.

Dr. Daniels was recipient of grant PF-725 from the American Cancer Society. Dr. Hamprecht was recipient of a grant from the Max-Planck Institute for Cell Chemistry.

Received for publication 23 February 1974, and in revised form 12 July 1974.

REFERENCES

1. AUGUSTI-TOCCO, G., and G. SATO. 1969. Establishment of functional clonal lines of neurons from mouse neuroblastoma. *Proc. Natl. Acad. Sci. U. S. A.* **64**:311.
2. SCHUBERT, D., S. HUMPHREYS, C. BARONI, and M. COHN. 1969. In vitro differentiation of a mouse neuroblastoma. *Proc. Natl. Acad. Sci. U. S. A.* **64**:316.
3. AUGUSTI-TOCCO, G., G. H. SATO, P. CLAUDE, and D. D. POTTER. 1970. Clonal cell lines of neurons. *Symp. Int. Soc. Cell Biol.* **9**:109.
4. AMANO, T., E. RICHELSON, and M. NIRENBERG. 1972. Neurotransmitter synthesis by neuroblastoma clones. *Proc. Natl. Acad. Sci. U. S. A.* **69**:258.
5. NELSON, P., W. RUFFNER, and M. NIRENBERG. 1969. Neuronal tumor cells with excitable membranes grown in vitro. *Proc. Natl. Acad. Sci. U. S. A.* **64**:1004.
6. NELSON, P. G., J. H. PEACOCK, T. AMANO, and J. MINNA. 1971 *a*. Electrogenesis in mouse neuroblastoma cells in vitro. *J. Cell. Physiol.* **77**:337.
7. HARRIS, A. J., and M. J. DENNIS. 1970. Acetylcholine sensitivity and distribution on mouse neuroblastoma cells. *Science (Wash. D.C.).* **167**:1253.
8. NELSON, P. G., J. PEACOCK, and T. AMANO. 1971 *b*. Responses of neuroblastoma cells to iontophoretically applied acetylcholine. *J. Cell. Physiol.* **77**: 353.
9. MINNA, J., D. GLAZER, and M. NIRENBERG. 1972.

Genetic dissection of neural properties using somatic cell hybrids. *Nat. New Biol.* **235**:225.

10. MINNA, J., P. NELSON, J. PEACOCK, D. GLAZER, and M. NIRENBERG. 1971. Genes for neuronal properties expressed in neuroblastoma × L cell hybrids. *Proc. Natl. Acad. Sci. U. S. A.* **68**:234.
11. AMANO, T., B. HAMPRECHT, and W. KEMPER. 1974. High activity of choline acetyl-transferase induced in neuroblastoma × glia hybrid cells. *Exp. Cell Res.* **85**:399.
12. KLEBE, R. T., T. CHEN, and F. H. RUDDLE. 1970. Controlled production of proliferating somatic cell hybrids. *J. Cell Biol.* **45**:74.
13. LITTLEFIELD, J. W. 1964. Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. *Science* (*Wash. D. C.*). **145**:709
14. BLUME, A., F. GILBERT, S. WILSON, J. FARBER, R. ROSENBERG, and M. NIRENBERG. 1970. Regulation of acetylcholinesterase in neuroblastoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **67**:786.
15. KARNOVSKY, M. J. 1967. The ultrastructural basis of capillary permeability studied with peroxidase as a tracer. *J. Cell Biol.* **35**:213.
16. BENDA, P., J. LIGHTBODY, G. SATO, L. LEVINE, and W. SWEET. 1968. Differentiated rat glial cell strain in tissue culture. *Science* (*Wash D. C.*). **161**:370.
17. RYTER, A., and PH. BENDA. 1972. Etude au microscope electronique d'hybrides de cellules gliales et de fibroblastes. *Exp. Cell Res.* **74**:407.
18. YAMADA, D. M., B. S. SPOONER, and N. K. WESSELS. 1971. Ultrastructure and function of growth cones and axons of cultured nerve cells. *J. Cell Biol.* **49**:614.
19. BUNGE, M. B. 1973. Fine structure of nerve fibers and growth cones of isolated sympathetic neurons in culture. *J. Cell Biol.* **56**:713.
20. TEICHBERG, S., and E. HOLTZMAN. 1973. Axonal agranular reticulum and synaptic vesicles in cultured embryonic chick sympathetic neurons. *J. Cell Biol.* **57**:88.
21. DAVIDSON, R. L. 1971. Regulation of gene expression in somatic cell hybrids: a review. *In Vitro.* **6**: 411.
22. FRYDMAN, R., and L. B. GELFEN. 1973. Depletion and repletion of adrenal dopamine-β-hydroxylase after reserpine. Immunohistochemical and fine structural correlates. *J. Histochem. Cytochem.* **21**: 166.
23. HAMPRECHT, B., J. TRABER, and F. LAMPRECHT. 1974. Dopamine-β-hydroxylase activity in cholinergic neuroblastoma × glioma hybrid cells; increase of activity by N^6,$O^{2'}$-dibutyryl adenosine 3',5'-cyclic monophosphate. *FEBS* (*Fed. Eur. Biochem. Soc.*) *Lett.* **42**:221.
24. HSIE, A. W., and T. T. PUCK. 1971. Morphological transformation of Chinese hamster cells by dibutyryl adenosine cyclic 3',5'-monophosphate and testosterone. *Proc. Natl. Acad. Sci. U. S. A.* **68**: 358.
25. JOHNSON, G. S., R. M. FRIEDMAN, and I. PASTAN. 1971. Restoration of several morphological characteristics of normal fibroblasts in sarcoma cells treated with adenosine 3',5'-cyclic monophosphate and its derivatives. *Proc. Natl. Acad. Sci. U. S. A.* **68**:425.
26. FURMANSKI, P., D. L. SILVERMAN, and M. LUBIN. 1971. Expression of differentiated functions in mouse neuroblastoma mediated by dibutyryl cyclic adenosine monophosphate. *Nature* (*Lond.*). **233**: 413.
27. PRASAD, K. N., and A. W. HSIE. 1971. Morphologic differentiation of mouse neuroblastoma cells induced in vitro by dibutyryl adenosine 3,5'-cyclic monophosphate. *Nat. New Biol.* **233**:141.
28. ROISEN, F. J., R. A. MURPHY, M. E. PICHICHERO, and W. G. BRADEN. 1972. Cyclic adenosine monophosphate stimulation of axonal elongation. *Science* (*Wash. D. C.*). **175**:73.