DOPAMINE-β-HYDROXYLASE ACTIVITY IN CHOLINERGIC NEUROBLASTOMA × GLIOMA HYBRID CELLS; INCREASE OF ACTIVITY BY N^6,$O^{2'}$-DIBUTYRYL ADENOSINE 3′:5′-CYCLIC MONOPHOSPHATE

Bernd HAMPRECHT and Jörg TRABER
Max-Planck-Institut für Biochemie, 8033 Martinsried, Germany

and

Friedhelm LAMPRECHT
Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Maryland 20014, USA

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1. Introduction

The last step in the biosynthesis of noradrenaline, the hydroxylation of dopamine, is catalized by the enzyme dopomine-β-hydroxylase (DBH, EC 1.14.2.1) [1]. As this enzyme is found only in adrenal glands and adrenergic nerves [2,3], it can serve as a marker for adrenergic neurons. The enzyme was also detected in neuroblastoma C 1300, a spontaneous tumor of the region of spinal cord of a mouse, and in clonal cell lines derived from it [4]. In hybrids between mouse neuroblastoma and rat glioma cells [5] dense core vesicles are observed [6]. They resemble those seen in adrenal medulla [7] or adrenergic nerves [8], which contain catecholamines [9,10] and DBH [11–13]. Therefore, it was tempting to assume that DBH and catecholamines were also present in the neuroblastoma × glioma hybrid cells.

In this communication we should like to report that DBH is found in 2 neuroblastoma × glioma hybrid cell lines and in the parental neuroblastoma cell line, but cannot be detected in the parental glioma line. The specific activity of DBH is much higher in the hybrid cells than in the neuroblastoma line. The hybrid cells also show high specific activities of choline acetyltransferase, the enzyme catalizing the biosynthesis of the neurotransmitter acetylcholine [5], and contain clear vesicles, which are a characteristic of cholinergic neurons [6]. Thus the cells simultaneously have characteristics of both cholinergic and adrenergic neurons. In the presence of N^6,$O^{2'}$-dibutyryl adenosine 3′:5′-cyclic AMP (dibutyryl cyclic AMP) the hybrid cells send out long processes. The specific DBH activity of one hybrid clone increases several fold above the control values when the cells are grown in the presence of this compound. Neither tyrosine hydroxylase (EC 1.14.3.a) [14], the enzyme catalizing the first step in the pathway from tyrosine to noradrenaline, nor dopamine or noradrenaline can be detected in the parental and the hybrid cell lines.

2. Materials and methods

2.1. *Cell culture*

The neuroblastoma cell line N18TG2 is a 6-thioguanine resistant mutant [15] of a clonal line derived from mouse neuroblastoma C-1300 [16]. The line C6-BU-1 is a bromodeoxyuridine resistant mutant [17] of clonal line C6 derived from a chemically induced tumor of a rat embryo [18]. Hybrids between lines N18TG2 and C6-BU-1 were prepared by fusion in the presence of inactivated Sendai virus as will be described elsewhere [5]. The two hybrid clones mentioned in this paper have the

laboratory designations 108CC5 and 108CC15. The cells were grown at 37°C in Falcon plastic flasks (75 cm^2) or plastic dishes (diameter 100 mm) as described [19]. The growth medium for N18TG2 was supplemented with 0.1 mM 6-thioguanine (Sigma), that for C6-BU-1 with 0.1 mM bromodeoxyuridine (Sigma) and that for the hybrid clones with 0.1 mM hypoxanthine (Sigma), 10 μM aminopterine (Nutritional Biochemical Corp.) and 0.16 mM thymidine (Sigma). In the cases indicated the medium contained 4 mM dibutyryl cyclic AMP (Sigma). The cells were subcultured by brief exposure to 0.05% trypsin [20]. They were counted in a hemocytometer and their viability was determined by exclusion of nigrosin.

2.2. *Assays*

After removal of the growth medium, the cells were washed twice with ice-cold medium D_2 [21] adjusted to 340 mOsmols [20]. After draining of the plates at an angle of approximately 45 degrees for 1 min, the cells were scraped off using ice-cold 0.05 M Tris–HCl buffer, pH 6.0, containing 0.2% Triton X-100. The cell suspensions were homogenized by sonication, the homogenates divided into several aliquots and stored over liquid nitrogen. A separate aliquot was thawed immediately before each assay and kept in ice. Tyrosine hydroxylase [22], DBH [23], dopamine and noradrenaline [24] were determined as described. Protein was assayed using bovine serum albumin as standard [25].

3. Results

On treatment with dibutyryl cyclic AMP for more than 8 days neuroblastoma × glioma hybrid clones extend long processes (fig. 1). Similarly, this compound induces formation of processes in glioma line C6-BU-1, though already within 24 hours [26]. When treated this way for several days the neuroblastoma

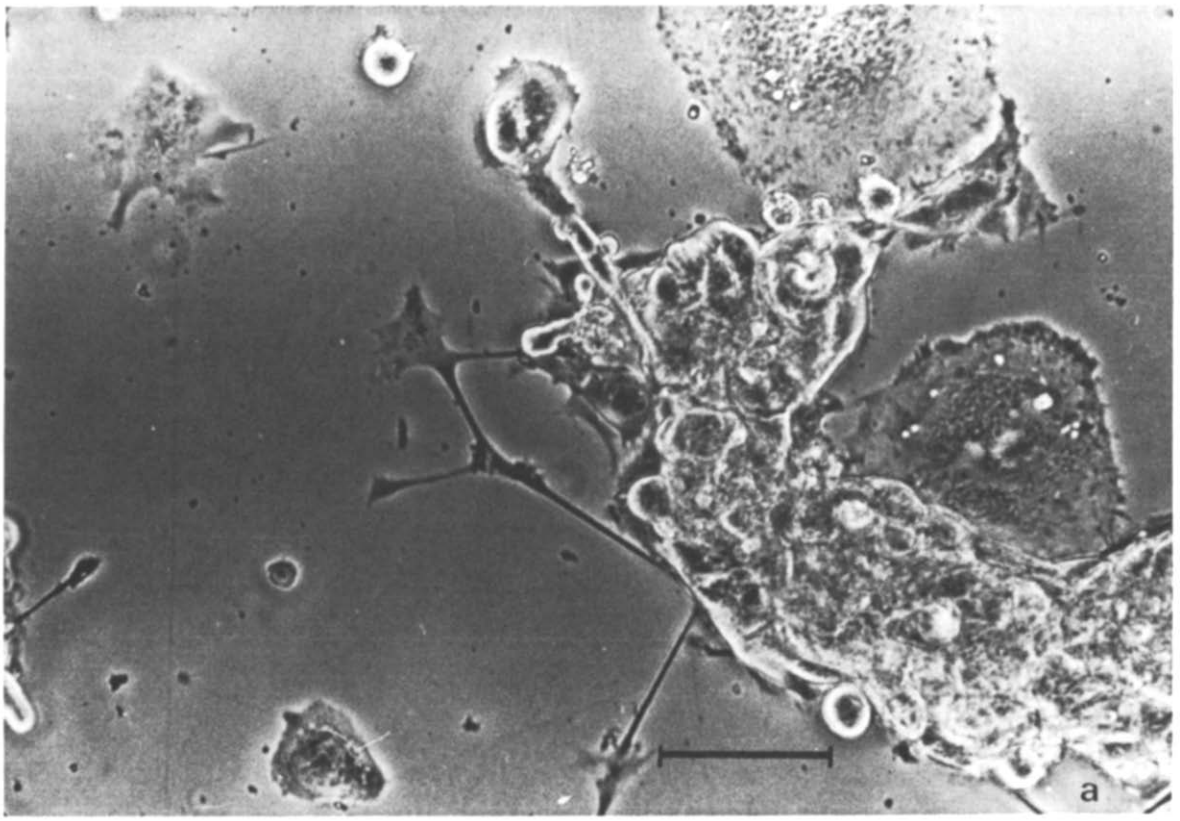


Fig. 1a. (Legend under 1c.)

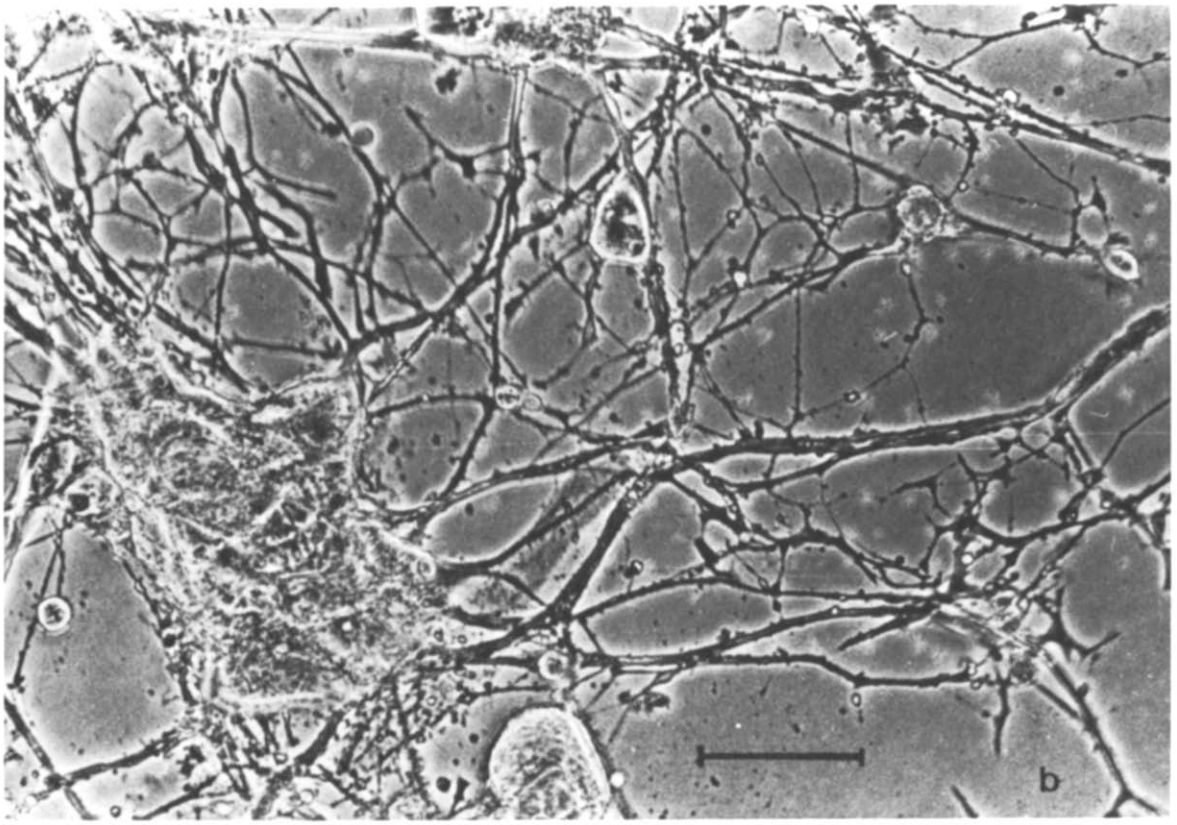


Fig. 1b. (Legend under 1c.)

cells N18TG2 form comparatively short processes.

The activity of DBH found is strongly dependent on the concentration of Cu^{2+} ions in the assay system [27]. Therefore, in order to determine the maximal activity of the enzyme, assays were performed at varying concentrations of the metal ion. Fig. 2 shows that the optimal concentration of cupric ions added to the assay mixture is lower for cells treated with dibutyryl cyclic AMP than for untreated cells. Neuroblastoma clone N18TG2 requires the addition of much higher concentrations of Cu^{2+} ions than the hybrid clones. The reason for these differences could be variations of cellular levels of free Cu^{2+} ions or of DBH inhibitors [27–30].

Irrespective of the maintenance in presence or absence of dibutyryl cyclic AMP, DBH activity cannot be detected in glioma line C6-BU-1 (table 1). In contrast, neuroblastoma line N18TG2 and both hybrid clones contain DBH activity. Comparing cells not treated with dibutyryl cyclic AMP, the specific activities of DBH are 3–5 times higher in the hybrids than in the parental neuroblastoma cells. On growth in the presence of dibutyryl cyclic AMP, specific activities of DBH are reduced by 75% in the neuroblastoma cells, remain constant in hybrid clone 108CC5 and increase by a factor of 3 in hybrid clone 108CC15 (table 1).

None of the cell lines tested (see table 1) contained tyrosine hydroxylase activity and, consistently, neither dopamine nor noradrenaline could be detected.

4. Discussion

The extension of processes in response to dibutyryl cyclic AMP has been reported for clonal lines of neuroblastoma [31] and glioma cells [26]. The processes of the neuroblastoma × glioma hybrids (fig. 1) are much thicker (up to 10 μ) and longer (up to 0.5 mm) than those reported for neuroblastoma cells [31].

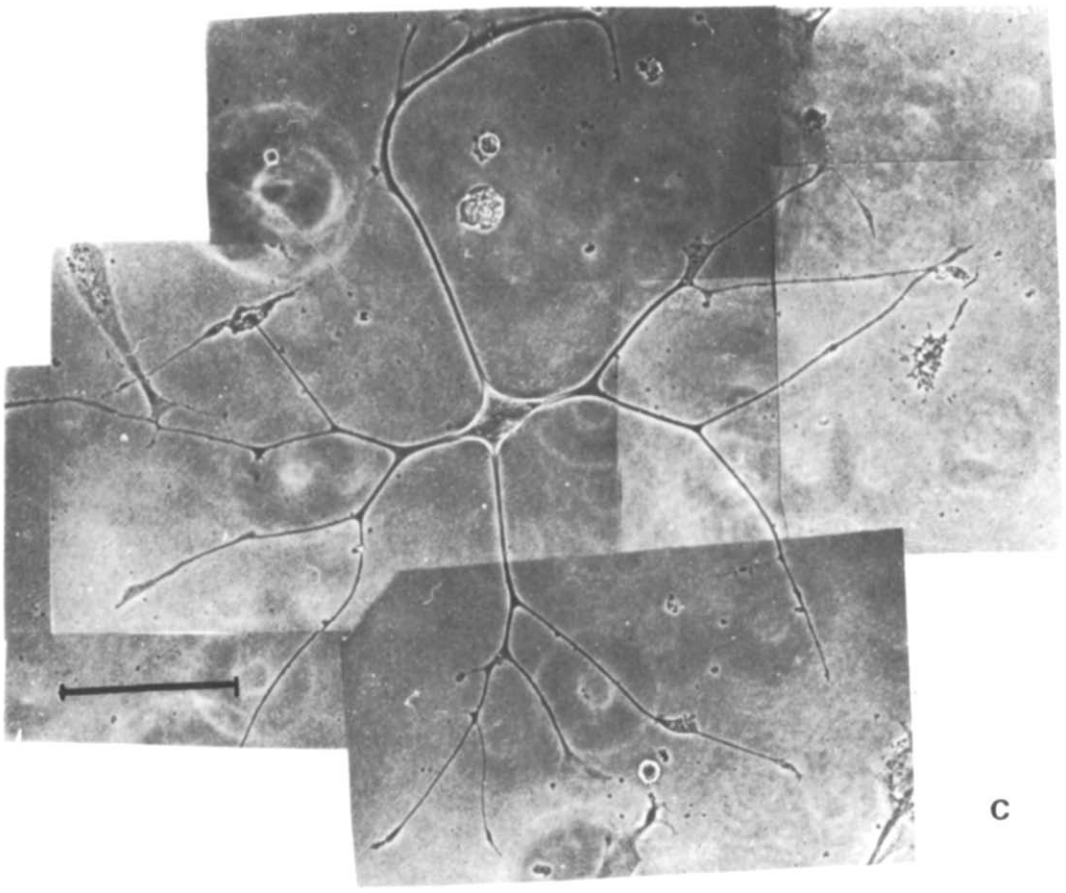
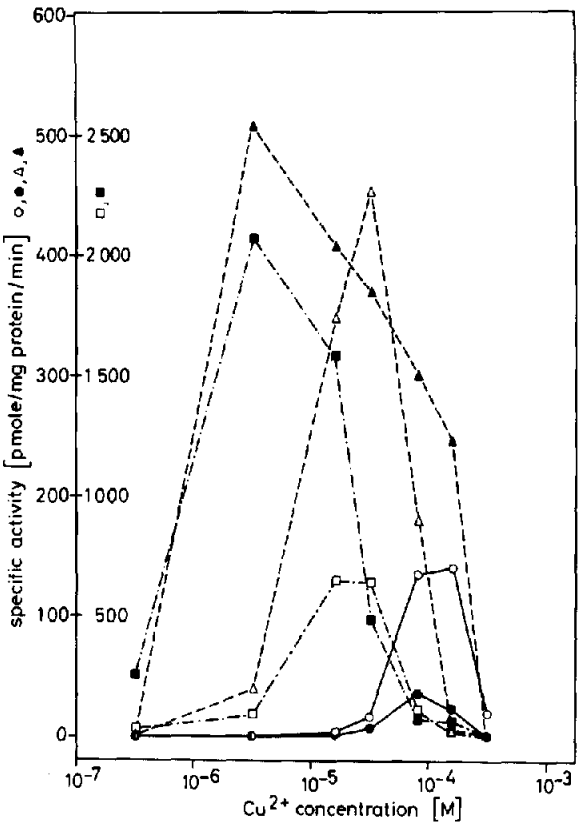


Fig. 1. Morphology of neuroblastoma × glioma hybrid clone 108CC5. (a) Cells grown in the absence of dibutyryl cyclic AMP. B, C. Two examples of cells treated with dibutyryl cyclic AMP. Cells were seeded, 2×10^4 (b) and 5×10^4 (c), respectively, per 60 mm plate, and 3 (B) and 4 (C) days later, respectively, the medium was replaced by the same medium supplemented with 4 mM (B) and 1 mM (C) dibutyryl cyclic AMP, respectively. The photomicrographs were taken 11 and 16 days, respectively, after the onset of the treatment with dibutyryl cyclic AMP. The black bar represents 100 μm in A–C. Note the binary branching of the processes in C.

It is consistent with the view of DBH as a marker of adrenergic neurons that the enzyme could not be detected in glioma cells, even not in those stimulated to extend processes. The specific activity of DBH in the mutant neuroblastoma line N18TG2 is comparable to that reported for the wild type line N18 [4]. The activity of choline acetyltransferase in N18TG2, though low, is well above the borderline of detectability [32]. In addition to DBH activity, N18TG2 cells contain dense core vesicles [6], a characteristic of adrenergic neurons that has already previously been observed with neuroblastoma cells [33]. However, as dense core vesicles are occasionally observed even in cholinergic nerve endings [34], their presence is not sufficient to characterize a cell as adrenergic. It is the simultaneous presence of DBH and dense core vesicles that justifies talking about adrenergic properties. Although it has not been firmly established by histochemical or immunohistochemical procedures that choline acetyltransferase and DBH are present in the same cell, the possibility of a mixture of 2 cell populations is unlikely, since clonal cell lines were used. Thus, N18TG2 is a cell line which simultaneously displays proper ties of cholinergic and adrenergic



neurons. The presence of cholinergic and adrenergic properties in the same cell is even much more striking in the glioma X neuroblastoma hybrid cells. These cells have high activities of choline acetyl transferase [5,32] and clear vesicles [6] as characteristics of cholinergic neurons, and DBH activities and dense core vesicles as characteristics of adrenergic neurons. However, the set of adrenergic properties is incomplete. Tyrosine hydroxylase, the first enzyme in the pathway of noradrenaline synthesis, is missing and, consequently, dopamine and noradrenaline are not being synthesized from tyrosine. As the cell homogenates were not assayed for the presence of dihydroxyphenylalanine decarboxylase, it is not known, whether or not the second enzyme of the pathway is also missing in the neuroblastoma and hybrid cells. To our knowledge these results show for the first time clearly the presence of cholinergic and adrenergic

Fig. 2. Relationship between the concentration of Cu^{2+} ions added to the assay mixture and DBH activity for neuroblastoma and hybrid cell lines. Open symbols, cells not treated; closed symbols, cells treated with 4 mM dibutyryl cyclic AMP. Circles: neuroblastoma N18TG2; triangles and squares: hybrid clones 108CC5 and 108CC15, respectively.

Table 1
Specific activity of DBH in neuroblastoma x glioma hybrids and their parent cells in absence and presence of dibutyryl cyclic AMP

Cell line	Time of treatm.* (days)	Total time of culture	Number of viable cells ($\times 10^{-6}$) inoculated	at harvest	Viability at harvest (%)	Optimal concentr. of Cu^{2+} (μM)	Spec. activity of DBH (pmoles·min^{-1} mg protein^{-1})
Neuroblastoma	0	5	4.8	50	86.3	167	140
N18TG2	4	13	0.024	2.8	96.4	83	37
Glioma	0	6	0.99	48	98.7	–	0
C6-BU-1	11	19	0.145	4.8	92.7	–	0
Hybrid	0	7	0.06	4.3	92.3	33	450
108CC5	10	13	0.048	0.060	85.7	3	510
Hybrid	0	7	0.028	3.7	90.8	17	650
108CC15	10	13	0.051	0.11	65.9	3	2070

* Treatment with 4 mM dibutyryl cyclic AMP.
The cells were grown in 100 mm dishes containing 20 ml of medium, except clonal line N18TG2 (absence of dibutyryl cyclic AMP), which was cultured in flasks (75 cm^2). The media were renewed every 1 to 4 days as required to prevent the media from becoming acidic.

markers in the same cell. They suggest that it might principally be possible to have genes expressed in the same cell which are coding for proteins of more than one neurotransmitter system (comprising biosynthesis, storage, release, re-uptake etc. of a neurotransmitter). In addition, the results demonstrate that not all genes of a given neurotransmitter system (e.g. the adrenergic system) must necessarily be expressed simultaneously. It remains to be established whether this is true only for the aneuploid cells used in this study or whether it holds true also for the euploid cells of nervous tissue.

As DBH was shown to be present in the dense core vesicles of adrenergic tissue [11–13,35,36], it is tempting to predict that this is also the case in the neuroblastoma and the hybrid cells. In the hybrid cells the formation of processes and the activity of DBH are regulated. Although dibutyryl cyclic AMP causes cells of both hybrid clones to extend large processes, it elevates the DBH activity in only one of them. This means that in the hybrid cells the extension of processes is not necessarily coupled with the increase in DBH activity.

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