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Clonal hybrid cell lines expressing cholinergic and adrenergic properties

[cell fusion/choline acetyltransferase/tyrosine hydroxylase (tyrosine 3-monooxygenase)/flow cytofluorimetry/microspectrofluorimetry]

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ABSTRACT **Different cholinergic cell lines were fused with an adrenergic neuroblastoma cell line (N115-BU-8). Its fusion with a cholinergic neuroblastoma–glioma hybrid produced a "hybrid-hybrid" line containing cholinergic and adrenergic enzyme activities. Both activities were also present in subclones of this line. The presence of catecholamines in single cells was confirmed by microspectrofluorimetry. These results are discussed with respect to the possibility of a simultaneous synthesis of noradrenaline and acetylcholine in single cells. The cholinergic and adrenergic enzyme activities are influenced by cell density, by dexamethasone, and by conditioned medium.**

On rigorous cloning of the mouse neuroblastoma C1300 three kinds of cell lines were found. They were cholinergic, adrenergic, or displayed neither property. A cell type exhibiting both activities was not found (1). Here we report that, by fusion of a cholinergic with an adrenergic cell line, hybrid cell lines can be obtained that are simultaneously cholinergic and adrenergic (2): they produce neurotransmitters of both types, acetylcholine (AcCho) and noradrenaline.

MATERIALS AND METHODS

Cells. Cholinergic and adrenergic cell lines (clones NS20Y and N1E-115) are derived from mouse neuroblastoma C1300 (1). Mouse neuroblastoma–rat glioma hybrid cells (clones 108CC15 and 108CC5) have been described (3, 4). 5-Bromodeoxyuridine-resistant as well as 6-thioguanine-resistant neuroblastoma mutants were produced by repeated treatment with ethyl methanesulfonate. Cells were then plated at various cell densities into solutions in which the drug concentrations were increased stepwise in the course of subcultivations, from 2 to 100 μM (clones NS20Y-TG, NS20Y-BU-7, N115-TG) and from 2 to 1000 μM (N115-BU-8), respectively (5).

Enzyme Assays. In all experiments, 10^6 viable cells were plated on plastic culture dishes 85 mm in diameter. After culturing for 4 days the cells were homogenized (5) and the specific activities of choline acetyltransferase (CAT; EC 2.3.1.6) (6) and tyrosine hydroxylase (TH; EC 1.14.16.2) (7) were determined as described, except that for the TH assay the incubation time was 30 min and the ion exchange columns contained a mixture of 20% Bio-Rad AG1-X8, OH^- form, and 80% AG50W-X8, H^+ form.

Flow Cytofluorimetry. A Fluvo-Metricell (8) was used. Cell suspensions were fixed with 70% (vol/vol) ethanol in water and stained for at least 30 min with mithramycin (100 μg/ml) dissolved in Puck's medium D1 containing 15 mM $MgCl_2$.

Thin-Layer Chromatography. For synthesis of AcCho, cells were incubated in 15 ml of modified Earle's salt solution (9) containing 10 μM choline iodide, 100 μM eserine, and [*methyl*-^{3}H]choline chloride at 4 μCi/ml (specific activity 0.39 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels). After extraction of cells (5 min) with 4 ml of methanol containing 0.1% acetic acid, the material was taken to dryness and redissolved in the phosphate buffer used in the CAT assay (6) [±20 units (as defined in ref. 6) AcCho esterase]. After incubation (20 min, 37°C) the material was applied to plates (cellulose F, Merck), which were developed with 1-butanol/acetic acid/H_2O (4:1:5 vol/vol; upper phase). R_F of AcCho, 0.47. For synthesis of dopamine and noradrenaline, cells were incubated (4 hr) in 2 ml of modified Earle's salt solution (9) containing L-[*U*-^{14}C]tyrosine at 4 μCi/ml (specific activity 486 mCi/mmol). The acid extract (0.2 M $HClO_4$, 10 min) was dansylated overnight as described (10). The mixture contained 200 μl of cell extract (corresponding to 1.5 mg of cellular protein), 600 μl of 1-dimethylaminonaphthalene-5-sulfonyl (dansyl) chloride (0.5 mg/ml) in acetone, 10 μl of 1 mM aqueous solution of each dopamine·HCl and noradrenaline bitartrate, and 100 mg of $Na_2CO_3 \cdot 10H_2O$. The catecholamines were extracted with 200 μl of benzene and then applied to plates (sil G-25 HR, Macherey & Nagel, Cologne, Germany). The spots were developed once in each of the perpendicular directions, using two solvent systems [ethyl acetate/cyclohexane (3:2 vol/vol), first direction; benzene/triethylamine (5:2 vol/vol), second direction]. R_F of noradrenaline, 0.52 and 0.29. R_F of dopamine, 0.61 and 0.64.

Spectrofluorimetry. Cells were treated for 5 min with 2% glyoxylic acid in 0.1 M sodium phosphate buffer, pH 7.0–7.3. The samples were then lyophilized for 4 days between −40°C and −50°C, heated for 6 min at 100°C, and measured in a dry chamber containing P_2O_5. Spectrofluorimetric measurements were performed with a Zeiss microscope equipped with the prism monochromator M4Q3 for selecting the excitation wavelength (bandwidth 10 nm) and with emission filters FT460 and LP495. The shift in the excitation spectrum was found to be dependent on the concentrations of catecholamines in dried films of protein. The shift was independent of the fluorescence intensity measured in the photometric field (a disc with a diameter of 12 μm). Details are given elsewhere (5).

RESULTS

Production of Cholinergic–Adrenergic Hybrid Cell Lines. In a first approach, bromodeoxyuridine-resistant (1 mM) as well as 6-thioguanine-resistant (0.1 mM) mutants of both a cholinergic (NS20Y) and an adrenergic (N1E-115) cell line were isolated (5). A mutant of the cholinergic line was fused (11) with the complementary mutant (12) of the adrenergic line. Hybrid

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Abbreviations: AcCho, acetylcholine; CAT, choline acetyltransferase; dansyl, 1-dimethylaminonaphthalene-5-sulfonyl; Bt_2cAMP, $N^6,O^{2'}$-dibutyryl cyclic AMP; HAT, hypoxanthine/aminopterin/thymidine; TH, tyrosine hydroxylase.

cells were selected in hypoxanthine/aminopterin/thymidine (HAT) medium (12) at a cell number insufficient for the occurrence of revertants. The isolated hybrid clones expressed only low specific activities of CAT (less than 25 pmol·min^{-1}·mg protein^{-1}) and TH (less than 7 pmol·min^{-1}·mg protein^{-1}). As controls, hybrid cells were generated by fusion of a mutant cholinergic or adrenergic cell line with the complementary mutant of the same cell line. These clonal hybrids had a tendency to lose CAT or TH activity, respectively, and never displayed both enzyme activities together (data not shown).

In a second approach, the adrenergic mutant (bromodeoxyuridine-resistant, also containing CAT of low specific activity) mouse neuroblastoma line N115-BU-8 (5) was fused with cholinergic wild-type cells. The latter were either neuroblastoma line NS20Y (1) or the mouse neuroblastoma–rat glioma hybrid lines 108CC5 or 108CC15 (3, 4). Besides carrying cholinergic markers such as CAT (3, 4) and AcCho (9), these hybrids are partially adrenergic (4) due to the presence of dopamine β-hydroxylase but not TH. After fusion, clones were isolated in HAT medium lacking tyrosine (13). They showed highly variable activities of CAT and TH (Fig. 1). In the case of the hybrid line 108CC15 as the cholinergic parent a hybrid-hybrid line (NH15-CA2) with high specific activities of CAT and TH was obtained (Fig. 1).

Line NH15-CA2 synthesizes AcCho and catecholamines from radioactive precursors. AcCho was identified by thin-layer chromatography and its sensitivity to AcCho esterase. Both dopamine and noradrenaline were identified as their dansyl derivatives by two-dimensional thin-layer chromatography (10) (data not shown).

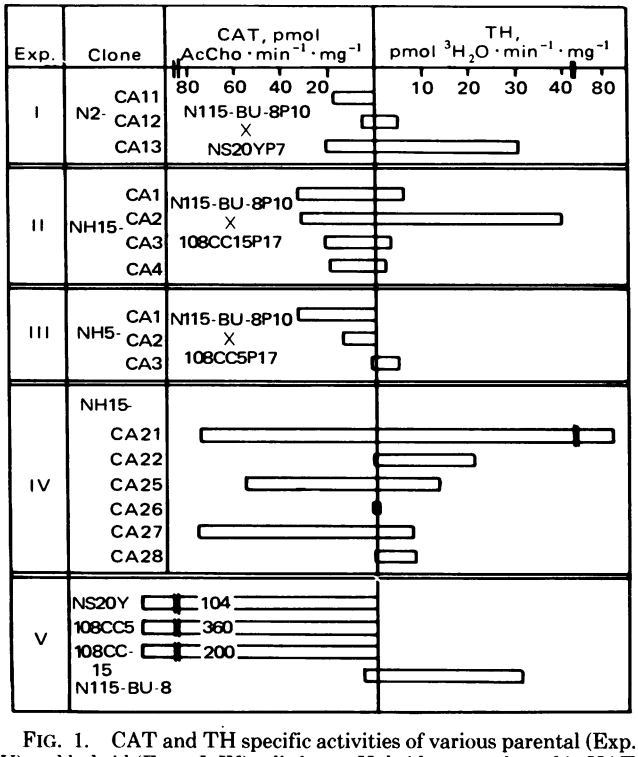

FIG. 1. CAT and TH specific activities of various parental (Exp. V) and hybrid (Exps. I–IV) cell clones. Hybrids were selected in HAT medium containing 10% fetal calf serum (4) (for Exps. I–IV dialyzed against Puck's medium D1) and lacking tyrosine (Exps. I–IV only). After isolated clonal hybrid cells (Exps. I–IV) had reached confluency, they were subcultured two to four times. Then cells were cultured in HAT medium for at least 4 days. The clones in Exps. I–III resulted from the fusion of the cell lines as indicated in the third column. P and a following number designate the number of subcultivations (passages). With the aid of cloning cylinders, six subclones of clone NH15-CA2P6 were picked from dishes (85 mm in diameter) containing one to three colonies (Exp. IV). In Exp. V the numbers in the bars represent specific CAT activities. The values of Exps. I–III were obtained from single plates; those of Exps. IV and V are mean values obtained from duplicate plates.

Subclones of NH15-CA2 grown in selective medium showed varying specific activities of CAT and TH, half of them resembling NH15-CA2 in the coexpression of CAT and TH (Fig. 1, experiment IV).

Hybrid-Hybrid Nature of Line NH15-CA2. Calibrated flow cytofluorimetry reveals that the DNA content of these cells is approximately the sum of that of the parental cells (Fig. 2). As a negative control, the parental cholinergic cells were fused among themselves and subjected to the selection and cloning conditions employed for clone NH15-CA2. No clone was obtained. As another negative control, the adrenergic mutant line N115-BU-8 was plated in the selective medium used for NH15-CA2. No revertant clone was found. Rat marker chromosomes (3, 4, 11) are detected in clone NH15-CA2. They must originate from the parental hybrid line 108CC15 (3, 4). These results suggest strongly that line NH15-CA2 has not been derived from two cells of the same parental line. Rather, it must have arisen from the fusion of a cholinergic and an adrenergic cell.

Microspectrofluorimetric Determination of Catecholamines in Single Cells. A specific shift in the excitation spectrum of glyoxylic acid-treated cells (Fig. 3) indicates high concentrations of catecholamines. After treatment with dexamethasone and N^6,$O^{2'}$-dibutyryl cyclic AMP (Bt_2cAMP), 67% of the 53 single cells measured were scored catecholamine-positive. The specific activity of CAT in this culture was 330 pmol·min^{-1}·mg protein^{-1}. The presence of catecholamines in 100% of the cells would have proven the coexpression of cholinergic and adrenergic activities in single hybrid-hybrid cells.

Regulation of CAT and TH Activities in the Hybrid-Hybrid Line NH15-CA2. TH specific activity increased with the cell density (Fig. 4*C*, curve b). This was also found in the wild-type precursor N1E-115 of the mutant line N115-BU8 (14) and for phenylalanine hydroxylase activity in hepatoma cells (15). The specific activity of CAT did not change with the cell density (Fig. 4*B*, curve b), as was also observed in neuroblastoma–glioma hybrid cells of high (ref. 16) but not of low (refs. 3, 4) passage number. In the presence of Bt_2cAMP the specific activities of CAT and TH were enhanced (Fig. 4 *B* and *C*, curves a). They were further influenced by dexamethasone or medium conditioned by polyploid cells C6-4-2 derived from rat glioma line C6 (5). During 5 days of incubation, dexamethasone (10 μM) raised the specific activity of CAT from 170 to 315 and that of TH from 190 to 580 (pmol·min^{-1}·mg protein^{-1}), while conditioned medium doubled only CAT specific activity.

DISCUSSION

Results from three different kinds of experiments could point to a coexpression of cholinergic and adrenergic activities in single hybrid-hybrid cells of line NH15-CA2: (*i*) Two-thirds of the single cells synthesize catecholamine as detected by microspectrofluorimetry. In the same culture high CAT activity is found. (*ii*) It has been possible to isolate subclones expressing both CAT and TH activities. (*iii*) In tyrosine-free medium hybrid-hybrid cells are selected that can produce tyrosine from phenylalanine due to catalysis by at least one of the three enzymes: phenylalanine hydroxylase, tryptophan hydroxylase, or TH (17). The presence of TH and catecholamine in line NH15-CA2 demonstrates that a substantial proportion of the cells was selected due to the presence of TH. One possible in-

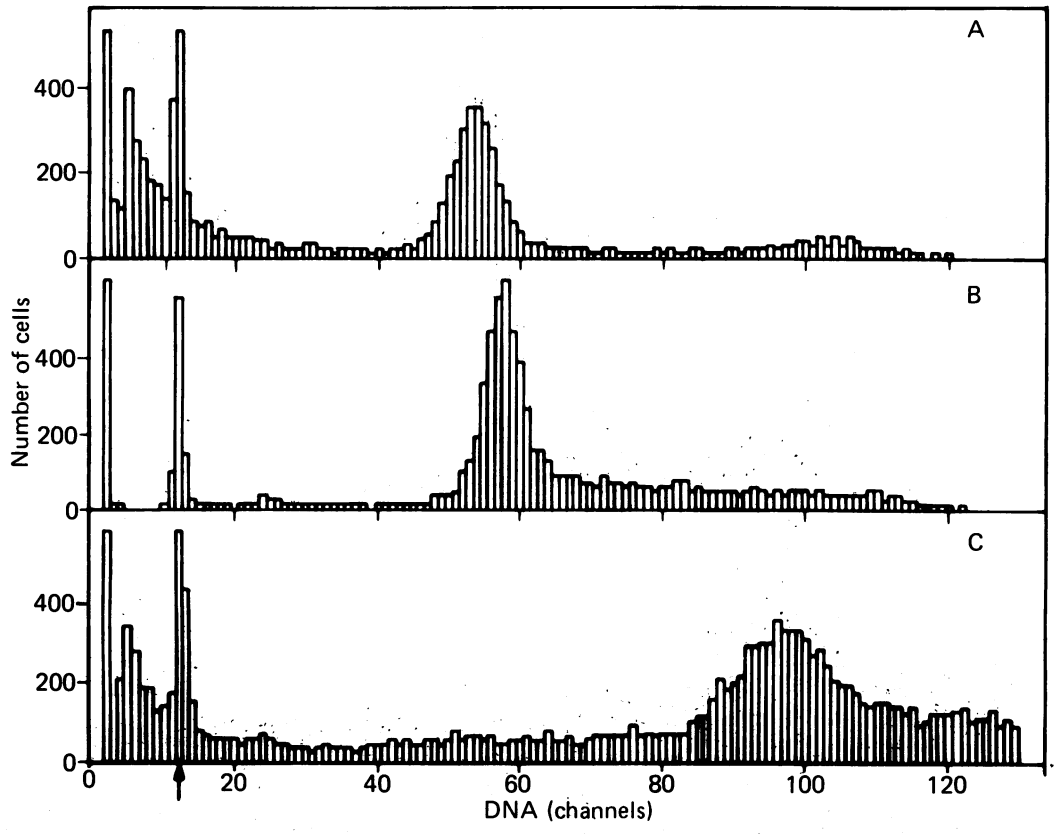

FIG. 2. Distribution curves for the cellular DNA content of line NH15-CA2 and its parental lines. The cells of the G1 populations of clones 108CC15P21 (*A*), N115-BU-8P22 (*B*), and NH15-CA2P7 (*C*) appear in channels 54, 58, and 96, respectively. Rat glioma cells of clone C6P65 were added for calibration (arrow, channel 12).

terpretation of the data presented is that adrenergic and cholinergic properties are simultaneously expressed in single cells. However, other explanations that are in accord with the data cannot be dismissed without carrying out further experiments. For example, we cannot exclude a possibility such as oscillation of individual cells between a cholinergic and an adrenergic state with the overall specific activities of the two enzymes remaining constant. Further experiments would have to aim at the simultaneous detection of cholinergic and adrenergic properties in single cells. They could involve fluorescence techniques for the detection of CAT and TH or CAT and catecholamines.

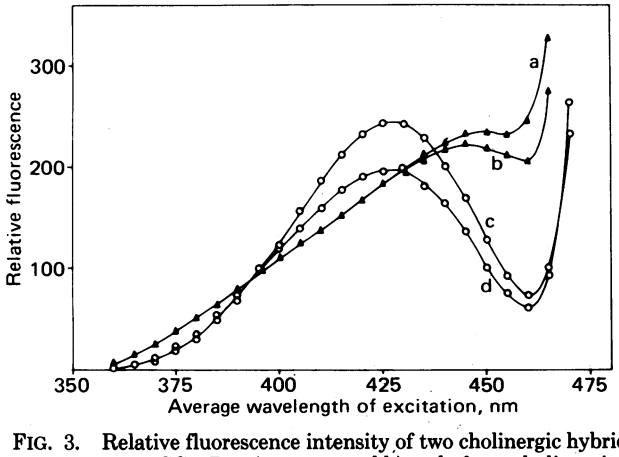

FIG. 3. Relative fluorescence intensity of two cholinergic hybrid cells of clones 108CC15P14 (curves a and b) and of two cholinergic–adrenergic hybrid-hybrid cells of clone NH15-CA2P7 (curves c and d) at various wavelengths of excitation.

Thus, antibodies would be required that can recognize CAT and TH in the hybrid-hybrid cell line. In this line these enzymes could be of rat or mouse origin or both (18). Obviously, it appears worthwhile to overcome the technical difficulties inherent in such experiments.

The presence of CAT and TH activities in neuroblastoma clones has been reported (19–23). However, these authors did not discriminate between CAT and carnitine acetyltransferase (6). Moreover, in comparison to the hybrid-hybrid cells, the specific activities were very low. The electrophysiologically detected release of AcCho and noradrenaline from a single cultured sympathetic ganglion cell is so far the only definite proof for the coexistence of the two neurotransmitters in a single cell (24). As in the case of the hybrid-hybrid cells, attempts to prove the synthesis of both neurotransmitters in the same cell were unsuccessful in the superior cervical ganglia (25), in pheochromocytoma cells (26), and in a giant neuron of a snail (27).

Regulation by conditioned media of cholinergic and adrenergic properties has been extensively studied in cultured sympathetic ganglia cells (28). Furthermore, in a rat pheochromocytoma line (29) both CAT and TH are regulated by various factors (30, 31), including conditioned medium in the case of CAT (31). It is not known whether the same conditioned medium factors are responsible for the effects in these two systems and in the hybrid-hybrid cells.

Because hybrid-hybrid cells can be grown as solid tumors in athymic *nude* mice, sufficient material for biochemical studies will be available (unpublished). The conservation of differentiated functions during passage through *nude* mice has already been shown for neuroblastoma–glioma hybrid cells (32). The hybrid-hybrid cells contain more than 200 chromosomes and

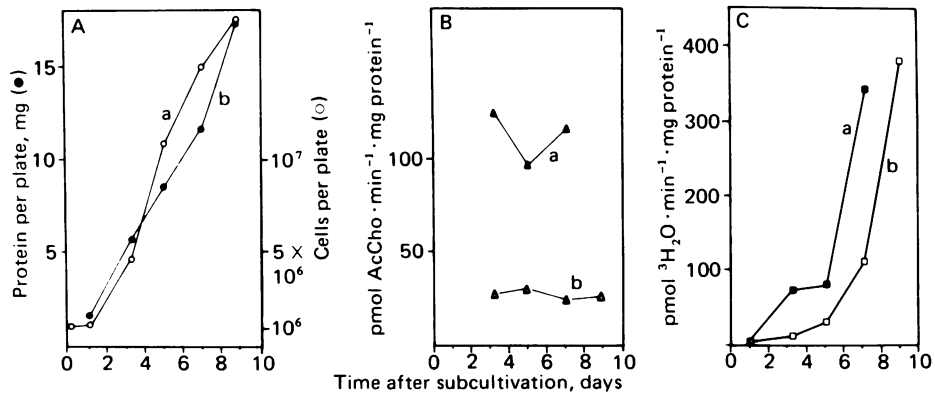

FIG. 4. Total number of cells (*A*, curve a) and mg of protein (*A*, curve b) per plate, and specific activity of CAT (*B*) and TH (*C*) as a function of the time after plating of hybrid-hybrid cells NH15-CA2 P12. In *B* and *C*, curves a are with 1 mM Bt_2cAMP since day 1; curves b are with no Bt_2cAMP. The points are data from single plates (*B* and *C*, curves a) or mean values of data from duplicate plates (other curves).

correspondingly (16) are large (diameter of detached cells: 24 μm). Their size facilitates intracellular electrical recording. Thus, as was found for the neuroblastoma–glioma hybrid cells (3, 4), they were found to be highly excitable, if adequately depolarized electrically or by AcCho (G. Reiser, personal communication). Like their parental hybrid cells (3, 4) they extend long neurite-like processes if treated with Bt_2cAMP. Their property of externally controlled simultaneous synthesis of AcCho and noradrenaline, their electrical excitability, their size, and their availability may make these cells valuable models in studying, e.g., neuron–glia interaction or innervation processes.

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