Correction. In the article “Human Phosphoribosylpyrophosphate Synthetase: Increased Enzyme Specific Activity in a Family with Gout and Excessive Purine Synthesis,” by Becker, M. A., Kostel, P. J., Meyer, L. J. & Seegmiller, J. E., which appeared in the October 1973 issue of the Proc. Nat. Acad. Sci. USA 70, 2749–2752, Fig. 1, p. 2750, was reproduced improperly by the printer. The figure and its accompanying legend are reprinted here. The investigation described in the article, was supported by grants AM 05646, AM 13622, and GM 17702 from the National Institutes of Health.

Fig. 1. Immunoprecipitation analysis of the reaction between purified human erythrocyte PP-ribose-P synthetase and rabbit serum. Center well contained 25 μl of 5000-fold purified normal PP-ribose-P synthetase (460 μg/ml). Numbered wells contained the following: wells 1 and 6, serum from immunized rabbits; wells 2 and 5, serum from unimmunized rabbits; wells 3 and 4, IgG fractions from unimmunized and immunized rabbits, respectively. Double diffusion was done for 24 hr at 4°C. Single precipitin bands are noted only where outer wells contained immunoglobulin from rabbits immunized with the purified enzyme.


Correction. In the article “Nucleotide Modification In Vitro of the Precursor of Transfer RNAγγ of Escherichia coli,” by Schaefer, K. P., Altman, S., and Soll, D., which appeared in Part 1 of the December 1973 issue of Proc. Nat. Acad. Sci. USA 70, 3628–3630, the following correction should be made. On p. 3628, the top line of the second column should read, “...found next to the anticodon (U0). In mature tRNA (18), in addition...”

Correction. In the article “Surface Antigens Common to Mouse Cleavage Embryos and Primitive Teratocarcinoma Cells in Culture,” by Artat, K., Dubois, P., Bennett, D., Condamine, H., Babinet, C. & Jacob, F., which appeared in the October 1973 issue of the Proc. Nat. Acad. Sci. USA 70, 2088–2092, Fig. 3, p. 2091, was reproduced improperly by the printer. It and its accompanying legend are reprinted here.

On page 2990 (first sentence of text, right-hand column) of the same paper, the sentence, “Absorptions were performed at 4°C for 30 min with 1 volume of C′:2 volumes of cells to diluted serum,” should read, “Absorptions were performed at 4°C for 30 min with 1 volume of packed cells to 2 volumes of diluted serum.”

Correction. In the article “Regulation of Acetylcholine Receptors in Relation to Acetylcholinesterase in Neuroblastoma Cells,” by Simantov, R. and Sachs, L., which appeared in the October 1973 issue of Proc. Nat. Acad. Sci. USA 70, 2902–2905, on page 2902 the beginning of line 10 in the second paragraph in Materials and Methods should read 500 Ci/mol instead of 500 Ci/m mole. On p. 2903, the left hand ordinate of Fig. 6 should read “No. of acetylcholine receptors/cell (× 10^{-6})” instead of “(× 10^{-7}).

Fig. 3. Peroxidase test on 129 morulae (magnification: ×800). (a) Preimmunization serum 1:800; (b) antiserum against F9, 1:800.
Regulation of Acetylcholine Receptors in Relation to Acetylcholinesterase in Neuroblastoma Cells

(Naja nigricollis snake venom/proteolytic enzymes/sulphydryl group-blocking compounds)

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Communicated by David Nachmansohn, June 26, 1973

ABSTRACT Purified α-toxin from Naja nigricollis snake venom labeled by [3H]acetylation binds specifically to the acetylcholine receptors of mouse neuroblastoma cells. Toxin binding was inhibited by inhibitors for nicotinic and muscarinic acetylcholine receptors. Clones of neuroblastoma cells were selected for low acetylcholinesterase (EC 3.1.1.7) activity with antibodies against this enzyme. Selection for an 80-fold decrease in acetylcholinesterase activity was not associated with any decrease in the number of acetylcholine receptors (3.4 × 10^6 per cell). Removal or inactivation of 80% of the acetylcholine receptors by proteolytic enzymes or by compounds that block sulphydryl groups did not change the activity of acetylcholinesterase on the cell surface. In addition to these results on the separation of acetylcholine receptors and acetylcholinesterase, a common regulation was found in that both the number of acetylcholine receptors and the activity of acetylcholinesterase were increased 5- to 10-fold when the cells stopped to multiply or were induced to differentiate by dibutyryl-cAMP. It is suggested that there are different genes for the acetylcholine receptor and acetylcholinesterase, and that both are regulated during growth and differentiation by a common regulatory gene.

Membrane excitation requires acetylcholine receptors and acetylcholinesterase (EC 3.1.1.7) (1, 2). Affinity-labeling procedures have indicated that there are similarities and differences between the active sites of the receptor and the enzyme (3-5). Acetylcholinesterase has been purified (6, 7), and purified acetylcholine receptors are reported to have no acetylcholinesterase activity (8-11). The receptor and the enzyme, both of which have sites specific for acetylcholine, may therefore be on different molecules, or there may be different sites on the same molecule that can be experimentally separated (2).

The present studies were undertaken to determine the regulation of acetylcholine receptors in relation to acetylcholinesterase in neuroblastoma cells, which can be cultured in vitro and used for genetic analysis. Using antibodies against partially purified acetylcholinesterase, we have selected clones with about a 100-fold difference in acetylcholinesterase activity (12). We have now characterized the acetylcholine receptors in these cells with purified α-toxin from Naja nigricollis snake venom labeled by [3H]acetylation. The relationship between acetylcholine receptors and acetylcholinesterase was studied in selected and unselected clones, after treatment of cells with proteolytic enzymes and compounds that block sulphydryl groups and during cell growth and differentiation.

ABBREVIATIONS: LD_50 and LD_100, dose at which 50 and 100%, respectively, of animals are killed.

MATERIALS AND METHODS

Cells, Cell Culture, and Induction of Differentiation. Mouse neuroblastoma C-1300 tumors in mice were obtained from Jackson Laboratories (Bar Harbor, Me.), and cell lines were established in culture. The cells were cloned and selected for decreased acetylcholinesterase activity by antibodies against acetylcholinesterase as described (12). Cells were subcultured and seeded for experiments at 2 × 10^6 cells per 50-mm tissue culture petri dish (Nunc, Denmark), unless otherwise stated, in Eagle's medium with a 4-fold concentration of amino acids and vitamins and 15% fetal calf serum. The clones used were numbers 1, 7, 7A, and 7D, with an acetylcholinesterase activity of 90.0, 16.2, 2.8, and 1.1 milliunits/mg of protein in the stationary phase of growth, respectively. For induction of differentiation, 10^5 cells were seeded per petri dish; and 24 hr after seeding dibutylryl-cAMP was added at a final concentration of 2 mM. The criterion used to define a differentiated cell was the existence of one or more processes larger than 50 μm.

Preparation of [3H]Toxin. α-Toxin was purified by filtration through a Sephadex G-75 column and two purifications by an Amberlite IRC-50 column (13). α-Toxin was labeled by acetylation with [3H]acetic anhydride in an anhydrous solid phase (14). 10 mg of α-toxin was lyophilized in a thin layer on the surface of the flask, and 2 ml of the following mixture was added: 10 ml of distilled benzene, 7 μl of acetic anhydride, and 150 μl of [3H]acetic anhydride (100 μCi/ml, specific activity 500 Ci/mmol). After 5 days of incubation at 4°C, the benzene was evaporated under reduced pressure and the material was filtered through a Sephadex G-25 column (50 × 0.9 cm) to remove free [3H]acetic anhydride. The specific activity of the [3H]acetylated-α-toxin was 40–80 Ci/mol. Since the specific activity of the [3H]acetic anhydride in the acetylation mixture was 150 Ci/mol, 25–50% of the amino groups of the toxin molecules were labeled. The median lethal dose (LD_50) and the total lethal dose (LD_100) of the [3H]toxin in mice were 2.5 and 3.4 μg, compared to 1.7 and 2.0 μg, respectively, for the non-labeled toxin.

[3H]Toxin Binding to Cells. Cells were collected from petri dishes with 0.02% ethylenediaminetetraacetic acid (EDTA) solution containing 8 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4, and 0.02 g of KH_2PO_4 in 1 liter of distilled water. The cells were washed twice with phosphate-buffered saline (pH 7.2), and 10^6 or 2 × 10^6 cells were incubated with the indicated amounts of [3H]toxin in 1 or 2 ml of phosphate-buffered saline. After 20 min of incubation at room temperature, 4 volumes of phosphate-buffered saline were added. The cells
were immediately centrifuged and washed twice with 5 ml of phosphate-buffered saline, and the radioactivity was counted after trichloroacetic acid precipitation on glass-fiber filters. For inhibition experiments, cells were preincubated for 20 min at room temperature in the presence of the inhibitor; [3H]toxin was added and the incubation was continued for a further 20 min. For treatment with proteolytic enzymes or compounds that block sulfhydryl groups, cells were preincubated at 37° for 5 or 10 min in the presence of the enzyme or the compound that blocks sulfhydryl groups, respectively. Four volumes of cold phosphate-buffered saline were then added and the cells were centrifuged and washed twice with phosphate-buffered saline. When cells were treated with trypsin, the reaction was stopped by addition of phosphate-buffered saline containing 50 μg/ml of soybean trypsin inhibitor; the cells were washed twice with the same solution, suspended in phosphate-buffered saline, and then assayed for binding of [3H]toxin. Unless otherwise stated, binding experiments were performed with cells in the stationary phase of growth 6–8 days after seeding.

Acetylcholinesterase Activity Was Determined in cell extracts as described by Blume et al. (15). 50 μl of reaction mixture, in 0.05 M phosphate buffer (pH 6.8) containing 0.2 M NaCl, 1 mM EDTA, 0.5% Triton X-100, and 3.3 mM [14C]-acetylcholine iodide (0.39 Ci/mmol). The reaction was stopped and the Dowex column was washed with 5 μM eserine sulfate solution. To test acetylcholinesterase activity on the cell surface of whole cells, the cells were washed twice with phosphate-buffered saline, and the enzyme assays were performed in phosphate-buffered saline instead of in 0.05 M phosphate buffer. Acetylcholinesterase activity on the surface of whole cells was about 90% of the activity in cell extracts.

Other Determinations. Cell-surface area was determined by centrifuging a known number of cells in centrifuge tubes containing a graduated capillary tube of 1-mm diameter (16). The amount of protein was determined by the method of Lowry et al. (17).

RESULTS

[3H]Toxin Binding to Neuroblastoma Cells. α-Toxin from the venom of Naja nigricollis was purified and labeled. Binding of [3H]toxin to neuroblastoma cells was 90% inhibited by nonlabeled toxin. The 10% apparently nonspecific binding of the [3H]toxin was also found with mouse fibroblasts (Fig. 1). The specific binding of [3H]toxin to neuroblastoma cells was inhibited by agonists and antagonists for the nicotinic acetylcholine receptors, decamethonium bromide, d-tubocurarine, carbamylocholine, nicotine, and acetylcholine (in the presence of 1 μM eserine sulfate to inhibit acetylcholinesterase activity) and also by the muscarinic inhibitors atropine sulfate and pilocarpine (Fig. 2 and Table 1).

The rate of [3H]toxin binding showed a plateau after 10 min (Fig. 3). From the linear part of the curve, the second-order rate constant was 1.1 to 1.8 × 10⁴ mol⁻¹ sec⁻¹. The number of [3H]toxin molecules bound to cells of clone no. 7 at saturation (Fig. 4) was 3.4 × 10⁴ per cell and 4.3 × 10⁴ per μm² of cell surface (Table 2). If one assumes nonreversibility of the reaction (10) and binding of one [3H]toxin molecule to one receptor molecule, this number is equal to the number of acetylcholine receptors per cell.

Number of Acetylcholine Receptors on Neuroblastoma Cells Selected for Low Acetylcholinesterase Activity. We have shown that antibodies against acetylcholinesterase can be used to select neuroblastoma clones with a decreased acetylcholinesterase activity (12). Clones no. 1 and 7, isolated without such selection, and clones 7A and 7D, selected with antibodies from

![Fig. 1. Inhibition of toxin binding to neuroblastoma cells by nonlabeled toxin. Neuroblastoma cells, clone no. 7 (○), and fibroblasts (●) were incubated with different concentrations of nonlabeled toxin for 20 min before 20 nmol of [3H]toxin was added.](image)

![Fig. 2. Specificity of [3H]toxin binding. Neuroblastoma cells, clone no. 7, were incubated for 20 min with acetylcholine (O), decamethonium bromide (△), or d-tubocurarine chloride (●) before addition of 20 nmol of [3H]toxin. 100% toxin binding was 66 pmol/mg of protein.](image)

<table>
<thead>
<tr>
<th>Compound added</th>
<th>μM</th>
<th>[3H]Toxin binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>1</td>
<td>95 ± 5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>35 ± 15</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>37 ± 3</td>
</tr>
<tr>
<td>Carbamylcholine</td>
<td>1</td>
<td>62 ± 3</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>48 ± 7</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>37 ± 3</td>
</tr>
<tr>
<td>Atropine</td>
<td>1</td>
<td>30 ± 10</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>10 ± 5</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>1</td>
<td>25 ± 15</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>20 ± 5</td>
</tr>
</tbody>
</table>

Cells were incubated with 20 nmol of [3H]toxin. 100% toxin binding was 66 pmol/mg of protein.
clone 7, were studied for the number of acetylcholine receptors in relation to acetylcholinesterase activity, in cells in the stationary phase of growth. The results (Table 2) show that all four clones had a similar number of acetylcholine receptors despite an 80-fold difference in acetylcholinesterase activity. Since clone 1 showed differences from clones 7, 7A, and 7D in attachment of cells to a tissue-culture petri dish (12), the clones were also tested in bacteriological petri dishes, in which none of the clones attached to the petri-dish surface. The same number of acetylcholine receptors and acetylcholinesterase activity per clone was found in cells grown in both types of petri dishes.

**Effect of Removal or Inactivation of Acetylcholine Receptors on Acetylcholinesterase Activity on the Cell Surface.** The relationship between the receptor and the enzyme on the cell surface was also studied by treatment of cells with proteolytic enzymes and compounds that block sulfhydryl groups. The results indicate (Table 3) that removal or inactivation of about 80% of the acetylcholine receptors by 5-min treatment with proteolytic enzymes or 10-min treatment with compounds that block sulfhydryl groups did not decrease the acetylcholinesterase activity on the cell surface.

**Regulation of the Number of Acetylcholine Receptors and Acetylcholinesterase Activity During Cell Growth and Differentiation.** The change from a linear to a stationary phase of growth (12, 15) and induction of differentiation by dibutyryl-cAMP (12, 18) is associated with an increase in acetylcholinesterase activity in neuroblastoma cells. Treatment of neuroblastoma cells with dibutyryl-cAMP, which increased acetylcholinesterase activity, decreased the activity of choline-O-

**DISCUSSION**

In the present experiments, we used α-toxin to quantitate and study the properties of acetylcholine receptors in neuroblastoma cells. Binding to these cells was specifically inhibited by nicotinic and muscarinic inhibitors of acetylcholine receptors. This result is in contrast to the acetylcholine receptors of cerebral cortex (19) and muscle (20, 21), which are only inhibited by nicotinic receptor inhibitors (19, 21). Our results with proteolytic enzymes and compounds that block sulfhydryl groups provide evidence that the acetylcholine receptor of neuroblastoma cells, like the acetylcholine receptors in normal cells (10, 19, 22), is, at least partially, a protein. The effect of compounds

**Table 2. Number of acetylcholine receptors and acetylcholinesterase activity in neuroblastoma clones**

<table>
<thead>
<tr>
<th>Clone no.</th>
<th>No. of acetylcholine receptors per cell</th>
<th>Acetylcholinesterase activity (milliunits/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg of protein</td>
<td>µmol² cell surface</td>
</tr>
<tr>
<td>1</td>
<td>31  (X10^{-4})</td>
<td>57  (X10^{-12})</td>
</tr>
<tr>
<td>7</td>
<td>34  (X10^{-4})</td>
<td>68  (X10^{-12})</td>
</tr>
<tr>
<td>7A</td>
<td>37  (X10^{-4})</td>
<td>74  (X10^{-12})</td>
</tr>
<tr>
<td>7D</td>
<td>35  (X10^{-4})</td>
<td>70  (X10^{-12})</td>
</tr>
</tbody>
</table>

Cells were tested in the stationary phase of growth.
that block sulfhydryl groups on acetylcholine receptors and the absence of any effect on acetylcholinesterase activity in neuroblastoma cells is also the same as that obtained with excitable membranes in normal cells (23-25).

Using antibodies against acetylcholinesterase, we selected neuroblastoma clones with an 80-fold decrease in acetylcholinesterase activity. This selection did not result in a decrease in the average number of $3.4 \times 10^7$ acetylcholine receptors per cell. This result suggests that there are different determinants for acetylcholine receptors and acetylcholinesterase. Proteolytic enzymes and compounds that block sulfhydryl groups removed or inactivated 80% of the acetylcholine receptors, without decreasing the activity of acetylcholinesterase on the cell surface. This further shows the experimental separation between the receptor and the enzyme in neuroblastoma cells. Our data also show that both acetylcholine receptors and acetylcholinesterase activity were increased when the cells stopped multiplying or were induced to differentiate. We suggest from the present results and the data on the purification of acetylcholine receptors from normal cells with no acetylcholinesterase activity (8-11), that there are different genes for the acetylcholine receptor and acetylcholinesterase and that both are regulated during growth and differentiation by a common regulatory gene.

We thank Mrs. Haya Dorf for skillful technical assistance.

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**Table 3. Effect of proteolytic enzymes and compounds that block sulfhydryl groups on the number of acetylcholine receptors and acetylcholinesterase activity**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mg/ml)</th>
<th>No. of acetylcholine receptors per cell ($\times 10^{-6}$)</th>
<th>Acetylcholinesterase activity* (milliunits/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>2.5</td>
<td>34</td>
<td>15.6</td>
</tr>
<tr>
<td>0.5</td>
<td>7</td>
<td>16.0</td>
<td></td>
</tr>
<tr>
<td>Pronase</td>
<td>5.0</td>
<td>8</td>
<td>15.7</td>
</tr>
<tr>
<td>0.5</td>
<td>10</td>
<td>14.5</td>
<td></td>
</tr>
<tr>
<td>α-Chymotrypsin</td>
<td>5.0</td>
<td>14</td>
<td>15.0</td>
</tr>
<tr>
<td>0.5</td>
<td>19</td>
<td>15.7</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 6.** Number of acetylcholine receptors and acetylcholinesterase activity during growth. Number of acetylcholine receptors (O), number of cells, (●), and acetylcholinesterase activity (△, milliunits/mg of protein), were determined at different days after $2 \times 10^7$ neuroblastoma cells from clone no. 7 were seeded per petri dish.