Detection of Nerve Growth Factor Binding Sites on Neuroblastoma Cells by Rosette Formation

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SUMMARY

A cloned suspension culture of mouse C1300 neuroblastoma cells bound, at 2°, sheep erythrocytes passively coated with nerve growth factor, with the formation of rosettes. When grown in tissue culture dishes to which they could attach, neuroblastoma cells rapidly transformed, within 48 hr, emitting cytoplasmic processes some of which were several mm long. Most of the attached neuroblastoma cells formed rosettes. In contrast, normal mouse kidney cells or various murine tumor cell lines used as cell controls exhibited a poor capacity for binding nerve growth factor. Rosette formation was a specific reaction that could be prevented by pretreating cells with proteolytic enzymes, free nerve growth factor, or specific antibodies against neuroblastoma cell extracts.

INTRODUCTION

NGF is a protein capable of producing rapid differentiation of normal sympathetic cells in vivo and in vitro (6). It has recently been shown that murine C1300 neuroblastoma cells, derived from a tumor of sympathetic cells (1, 8), bind on their membrane surface the NGF molecule with the same high avidity as normal sympathetic cells (2, 9, 10). This report describes a method for detecting NGF-specific membrane receptor sites by measuring the capacity of neuroblastoma cells to bind SRBC passively coated with NGF, with the formation of rosettes.

MATERIALS AND METHODS

Cells. A cell culture of murine C1300 neuroblastoma cells was kindly supplied by Dr. F. Jacob, Institut Pasteur, Paris, France. The cells were monocloned in our laboratory, and the clone designated NB/1R was used in these studies. The cells of this clone were homogeneous with respect to time division, morphology, staining characteristics, and sedimentation (9–11). Cultures were grown in suspension in 80-mm bacteriological Petri dishes, in EDM (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 20% fetal bovine serum (Flow Laboratories, Inc., Ayrshire, Great Britain). They were incubated at 37° in a humidifier incubator with 12% CO₂ and air. Under these conditions, the cells had an anaplastic round form (Fig. 1). When the cells were transferred to Falcon tissue culture dishes in medium without serum, they attached within 24 hr and emitted from the cell body cytoplasmic processes, some of which reached 1 to 2 mm in length (Fig. 2).

Mouse L929 cells (derived from NCTC, clone 929) were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum (Flow Laboratories) and 10% trypsin soy broth (Difco Laboratories, Detroit, Mich.).

Sarcoma 180 (from American Type Culture Collection) cells were maintained in Eagle's minimum essential medium containing 5% fetal calf serum, penicillin, streptomycin, and nonessential amino acids (10).

Sarcoma 25/37 cells, established in cell culture in our laboratory from murine Swiss-Webster ascitic tumor S/37, were grown in Roswell Park Memorial Institute (1640 medium supplemented with 10% calf serum, penicillin, and streptomycin (9).

Suspensions of primary kidney cells were prepared from Swiss-Webster mice immediately before use.

Before use, the above-mentioned cells were washed 3 times in EDM and their viability was checked by erythrosin dye (0.04%) exclusion. Cells were synchronized according to a modification (9) of the method of Tobey and Ley (14). By this procedure, more than 80% of the cells were synchronized in the G₁ phase of their growth cycle.

NGF. Mouse salivary gland NGF was isolated as a 27,500 m.w. dimer from Swiss-Webster mice by the method described by Bocchini and Angeletti (3). The purity of each NGF preparation was controlled by acrylamide gel electrophoresis in sodium dodecyl sulfate, performed as described by Shapiro et al. (13). The biological activity of the NGF preparations was tested by the method of Levi-Montalcini (6). Ten ng of this protein were capable of provoking in vitro, within 12 hr, the formation of a halo of nerve fibers from chick or mouse embryonic sensory and sympathetic ganglia.

Antibodies. Antibodies specific to neuroblastoma cell extracts were prepared in rabbits as follows. Neuroblastoma...
cells (10⁶) were homogenized in the cold with a glass homogenizer and a Teflon pestle (20 strokes) in 0.1 M sucrose, 0.01 M MgCl₂, and 0.1 M sodium phosphate buffer, pH 7.4. The cell debris was centrifuged at 1500 x g for 10 min. One mg protein of the supernatant was emulsified in complete Freund’s adjuvant (Difco) and injected into the 4 footpads of 2 rabbits; 2 weeks later, the rabbits received an i.m. inoculation of 1 mg protein from the same cell preparation, again in complete Freund’s adjuvant. This was followed at weekly intervals by 3 intradermal injections of 150 μg protein in 0.9% NaCl solution. When an Arthus reaction was visible, the rabbits were bled and the sera were pooled. The serum was absorbed by incubation at 4° with normal viable kidney, spleen, and liver cells from outbred mice. After precipitation with ammonium sulfate at 37% final saturation, the antibody content of the resulting globulin fraction was 150 μg antibody protein per ml.

Hyperimmune rabbit antiserum globulins and antiferritin antibodies were prepared in a similar manner. The antibody content of these preparations were, respectively, 880 and 1350 μg antibody protein per ml.

Chemicals. Trypsin (bovine pancreas), neuraminidase (Vibrio cholerae), cytochrome c, insulin, RNase (bovine pancreas), bacterial protease, and phospholipase C and D were purchased from Worthington Biochemical Corp., Freehold, N. J.; vinblastine (Velbe), was from Eli Lilly and Co., Indianapolis, Ind.; colchicine, sodium azide, and EDTA were from Sigma Chemical Co., St. Louis, Mo.; glutaraldehyde (50%, w/v) was from Fluka AG Chemische Fabrik, Buchs, Switzerland; formalin, ethanol, and phenol were from Merck & Co., Inc., Rahway, N. J.; and antimycin (crystalline) was from Difco.

NGF Coating to SRBC. SRBC in Alsever’s solution were washed 3 times in 0.05 M potassium phosphate-0.15 M NaCl, pH 7.4, and then fixed for 5 min at room temperature in 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. SRBC were then washed exhaustively, and 0.5 ml of packed cells was incubated in 5 ml of this buffer containing 5 mg highly purified NGF and 10 mg 1-ethyl-3-(3 diethylaminopropyl)-carbodiimide-hydrochloride (Ott Chemical Co., Muskegon, Mich.). As controls, SRBC were coated with unrelated proteins, such as cytochrome c, insulin, RNase, and mouse or rabbit γ-globulins. Other controls were comprised of similarly prepared cell suspensions without the addition of the protein to be tested. After 1 hr of incubation at room temperature, the cells were washed in 0.05 M potassium phosphate-0.15 M NaCl, pH 7.4, until no protein was detectable in the supernatant by absorbance readings at 280 nm. These erythrocytes did not spontaneously agglutinate and were stable for more than 20 days when tested by a passive hemagglutination test, using standard preparations of specific hyperimmune rabbit antibodies.

Test for Rosette-forming Cells. Cells in suspension were washed 3 times in serum-free medium or in 0.05 M potassium phosphate-0.05 M NaCl, pH 7.4. They were resuspended at a final concentration of 2.5 x 10⁶ cells/ml. Cell viability was always greater than 95% as measured by erythrosin dye exclusion. The presence of NGF-binding sites on neuroblastoma cells was investigated using synchronized neuroblastoma cells in suspension or using cultures of cells on coverslip. In the 1st test, 5, 1, or 0.5 x 10⁶ NGF-coated SRBC were added to 2.5 x 10⁴ nucleated cells in a final volume of 0.5 ml of 0.05 M potassium phosphate-0.15 M NaCl, pH 7.4, or EDM without serum. The suspension was incubated in glass tubes immersed in an ice bath for 5 min. The tubes were centrifuged in the cold for 5 min at 750 x g, and the cells were resuspended 10 min later in their own supernatant. The cells were examined microscopically for rosette formation at x 400. Cells with 5 or more adherent red cells were considered rosette-forming cells.

In the 2nd test, neuroblastoma cells were grown on coverslips in Leighton tubes in EDM without serum. After 24 to 48 hr, adherent cells were rinsed with 0.05 M potassium phosphate-0.15 M NaCl, pH 7.4, buffered saline and exposed to NGF- (or other protein) coated SRBC at room temperature. After 15 to 30 min of incubation, the coverslips were carefully removed from the tubes, held in a vertical position, and rinsed with 20 ml of 0.05 M potassium phosphate-0.15 M NaCl, pH 7.4, or medium. They were then examined microscopically.

Inhibition of Rosette Formation. Inhibition of rosette formation was investigated by exposing triplicate samples of neuroblastoma or other nucleated cells for 30 min at 2° to the following: normal rabbit γ-globulins, rabbit antineuroblastoma cell extract-antibodies, rabbit antiserum γ-globulins or antiferritin antibodies, sodium azide, phenol, 2-mercaptoethanol, formalin, glutaraldehyde, ethanol, and sodium azide, phenol, and various pH values, various concentrations of antimycin, soluble extracts of neuroblastoma or other cells, and various enzymes. Hyperimmune rabbit anti-neuroblastoma cell extract, antimouse γ-globulin, and antiferritin antibodies, all diluted to the same final protein concentration, were incubated in a final volume of 0.5 ml of medium with 2 x 10⁶ neuroblastoma cells.

Trypsin (150 μg protein per ml) was dissolved in 0.05 M potassium phosphate-0.15 M NaCl, pH 7.4, and incubated with 2.5 x 10⁶ cells at 37° for 30 min. Then an equimolar concentration of soybean trypsin-specific inhibitor (Worthington) was added to terminate the reaction. Neuraminidase (100 μg/ml, dissolved in calcium acetate buffer, pH 6.2) was incubated with cells at 37° for 30 min; then the pH was adjusted to 7.4 to prevent further enzymatic action. Bacterial proteases (100 μg/ml) were similarly incubated with the same number of cells in 0.05 M potassium phosphate-0.15 M NaCl, pH 7.4, or medium for 30 min at 37°. Phospholipase C and D (250 μg/ml) were dissolved in phosphate buffer (with CaCl₂ for phospholipase D); the pH of the reaction was maintained at about 6.2. After incubation for 30 min, the cells were washed 3 times with 2 ml 0.05 M potassium phosphate-0.15 M NaCl, pH 7.4, or medium. Cell viability was determined by dye exclusion, and if it was greater than 90 to 95%, the cells were tested for their capacity to form rosettes.

RESULTS

Rosette-forming cells. Neuroblastoma cells in suspension were synchronized and tested for rosette formation during

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the late G₁ phase of the cell cycle, because during this time there is maximal exposure of appropriate receptor sites on the cell membrane surface (9, 10). The synchronized cells formed rosettes when incubated with NGF-coated SRBC (Fig. 3). The rosettes appeared to be very stable since vigorous pipetting did not reduce the number of attached erythrocytes. Neuroblastoma cells in monolayer culture for 48 hr also exhibited high NGF-binding capacity: NGF-coated SRBC were firmly attached to the membrane of their cell body and along the network of axon-like processes (Fig. 4).

In contrast, under the same experimental conditions, normal mouse kidney cells and several unrelated murine tumor cell lines such as L929, 2S/37, and Sarcoma 180 exhibited a very poor capacity for binding NGF-coated SRBC on their surface membrane, even when synchronized in the G₁ phase of their growth cycle. Furthermore, the erythrocytes bound to these latter rosette-forming cells could generally be easily detached after 1 or 2 pipettings.

Rosette formation was largely dependent on the number of NGF-coated SRBC added to the reaction mixture and on the amount of NGF molecules coated onto each erythrocyte.

When extensively coated SRBC (10 mg NGF per 0.5 ml of packed SRBC) were used, the ratio of erythrocytes to neuroblastoma cells required to obtain 50% rosette-forming cells was 50 to 1.

In contrast, with lightly coated SRBC (0.5 mg NGF per 0.5 ml of packed SRBC), the ratio required was greater than 200 to 1. Under these different conditions, the number of rosette-forming cells among the cell controls fluctuated similarly but was always insignificant (less than 5%). In further experiments it then seemed feasible to use a preparation of NGF-coated SRBC that kept the number of rosettes among the controls as low as possible, but which was sufficiently labeled to allow saturation of rosette formation by synchronized neuroblastoma cells within the standard incubation time. This was achieved, as described in "Materials and Methods," by coating 0.5 ml of packed SRBC with 5.0 mg of NGF. Under these conditions, at a ratio of 200 SRBC per neuroblastoma cell, 80 to 85% of the neuroblastoma cells were rosette-forming cells. The number of rosette-forming cells decreased to about 50 to 60% at a ratio of 40 erythrocytes per single neuroblastoma cell and to about 30 to 50% at a ratio of 20 erythrocytes per neuroblastoma cell. The percentages of rosette-forming cells among the cell controls under these different conditions were 5, 3, and 1, respectively.

All experiments were then done with 5 × 10⁶ SRBC coated under these conditions and 2.5 × 10⁴ viable nucleated cells.

**Time-Course Kinetics of Rosette Formation.** A time-course kinetic study of rosette formation revealed that the binding of SRBC coated with NGF by neuroblastoma cells was a very quick and stable reaction, and 50% of the neuroblastoma cells were rosette-forming cells within 2 min of incubation at 2°. On the other hand, L929 cells were 50% rosette-forming cells only after 150 to 160 min and 2S/37, Sarcoma 180, or normal kidney cells were 50% only after several hr (Chart 1).

![Chart 1. Time course kinetics of rosette formation by murine C1300 neuroblastoma (A), L929 (O), 2S/37 and S(180 (C) cells, and a suspension or normal mouse kidney cells (——)).](chart1.png)

**Table 1.** Effect of different physical and chemical treatments on rosette formation at 2° by murine C1300 neuroblastoma cells and SRBC passively coated with NGF.

<table>
<thead>
<tr>
<th>Agents</th>
<th>Suspension</th>
<th>Monolayer</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>0.005 M</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>0.01 M</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Sodium azide, 0.1%</td>
<td>5</td>
<td>3</td>
<td>Damaging agents</td>
</tr>
<tr>
<td>Phenol, 0.3%</td>
<td>3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Merthiolate, 0.05%</td>
<td>30</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Buffers (4 &lt; pH &gt; 9)</td>
<td>98</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>Formalin, 0.5%</td>
<td>22</td>
<td>15</td>
<td>Fixative agents</td>
</tr>
<tr>
<td>Ethanol, 1%</td>
<td>28</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Glutaraldehyde, 1%</td>
<td>14</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Temperature, 4°</td>
<td>0</td>
<td>0</td>
<td>Cell respiration inhibitors</td>
</tr>
<tr>
<td>Antimycin, 0.1 mM</td>
<td>6</td>
<td>3</td>
<td></td>
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* Average triplicate tests on neuroblastoma cells grown in suspension or in monolayer.
### Table 2

<table>
<thead>
<tr>
<th>Agents</th>
<th>% inhibition(^a)</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuraminidase (100 µg/ml)</td>
<td>6</td>
<td>Sialic acid cleavage</td>
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<tr>
<td>Phospholipase C (250 µg/ml)</td>
<td>19</td>
<td>Phospholipid cleavage</td>
</tr>
<tr>
<td>Phospholipase D (250 µg/ml)</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Bacterial protease (100 µg/ml)</td>
<td>100</td>
<td>Proteolysis</td>
</tr>
<tr>
<td>Trypsin (150 µg/ml)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Colchicine (0.1 mM)</td>
<td>2</td>
<td>Microtubule assembly</td>
</tr>
<tr>
<td>Vinblastine (0.2 mM)</td>
<td>14</td>
<td>Microfilament contraction inhibitor</td>
</tr>
<tr>
<td>Cytochalasin B (0.04 mM)</td>
<td>3</td>
<td>Coating to membrane</td>
</tr>
<tr>
<td>Antiferritin antibodies(^b)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Anti-neuroblastoma extract antibodies(^b)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Neuroblastoma cells extract (100 µg/protien ml)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Free NGF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 µg/ml</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>1.0 µg/ml</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>10.0 µg/ml</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>100.0 µg/ml</td>
<td>100</td>
<td></td>
</tr>
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</table>

\(^a\) Average of triplicate tests on neuroblastoma cells grown in suspension or in monolayer.

\(^b\) Diluted to the same final protein content of 100 µg per ml antibody protein per ml.

DISCUSSION

These experiments clearly indicate that NGF binds at 2°C with firm bonds to the membrane surface of murine C1300 neuroblastoma cells. The interaction is mediated by appropriate receptor sites exposed on the cell surface membrane. These surface structures are probably of a protein nature, since rosette formation was strongly inhibited by pretreatment of cells with proteolytic enzymes but not with neuraminidase or phospholipases. Binding of NGF does not depend upon divalent cations and proceeds at low temperature, independent of the presence of azide, antimycin, or other drugs capable of preventing active metabolic processing by the cells. Cell fixation, provided it does not denature the structural conformation of appropriate receptor sites, has no effect on rosette formation, suggesting that these binding molecules are exposed primarily on the membrane.

Rosette formation was inhibited by preexposing the cells to hyperimmune antibodies specifically made against neuroblastoma cell extracts. Nonrelated antibodies or equal amounts of normal γ-globulins had, on the contrary, no inhibitory effect. Colchicine and vinblastine, 2 alkaloids that bind to microtubule protein subunits preventing their polymerization, did not inhibit the binding to neuroblastoma cells of NGF-coated SRBC. Likewise, no blocking effect was obtained with cytochalasin B, which is a strong inhibitor of microfilament contraction (15).

The question now arises as to the cell specificity of the NGF receptor molecules exposed on the surface membrane of neuroblastoma cells. The experiments demonstrate that binding of NGF to normal mouse kidney cells or to murine tumor cell lines does occur, but under the experimental conditions used it is almost insignificant. A close analysis of the time course kinetics of NGF binding to these cells shows that more and more "nonspecific" rosette-forming cells can be obtained by prolonging the incubation time. In addition, it should be noted that rosette formation in all the cells examined can be inhibited by preexposing the cells to antibodies made specifically against neuroblastoma cells. These findings suggest several possibilities. One is that a common receptor molecule for NGF is present in all these cells, but is at a higher concentration and/or is more exposed on the surface of neuroblastoma cells than on the surface of normal sympathetic nerve cells as

no inhibition induced by preexposing neuroblastoma cells to normal γ-globulins of rabbits or to rabbit antibodies directed against unrelated antigens, whereas a specific inhibitory effect was obtained with antibodies directed against neuroblastoma extract. Free NGF added in competition inhibited rosette formation, whereas nonrelated ligands of different molecular weight or charge had no effect even when added in high doses. Finally, vinblastine and colchicine, 2 inhibitors of microtubule polymerization, and cytochalasin B, a strong inhibitor of microfilament contraction, had no inhibitory effect on rosette formation by NGF-coated SRBC. Similar effects on inhibition of rosette formation were obtained with the normal murine kidney or tumor cell lines used as cell controls.
well. Extensive studies during recent years have, in fact, demonstrated a number of striking similarities in the biological and morphological properties of neuroblastoma cells and normal sympathetic nerve cells (1, 4, 5, 8, 11, 12). Although the latter respond to NGF by differentiating, only a few human neuroblastoma cell lines seem to be capable of this response to NGF (L. Bertolini, L. Diamond and R. Revoltella, unpublished observations). The present results show that our murine C1300 neuroblastoma cell line has in common with normal sympathetic cells the ability to bind the NGF molecule on its cell surface (2, 10), suggesting that neuroblastoma cells may be useful for studying induction of differentiation by NGF.

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