SPECIFIC BINDING OF NERVE GROWTH FACTOR (NGF) BY MURINE C 1300 NEUROBLASTOMA CELLS

By R. REVOLTELLA, L. BERTOLINI, M. PEDICONI, and E. VIGNETI

(*From the Laboratory of Cell Biology, Consiglio Nazionale delle Ricerche, Rome, Italy*)

(Received for publication 26 November 1973)

Mouse C 1300 neuroblastoma (NB)[1] is a tumor of sympathetic cells which grows in vitro in suspension as anaplastic round cells (1). When clones of these cells are plated on a surface to which they can attach, the cells differentiate and assume many of the morphological and biochemical properties of mature sympathetic cells (2).

There has been intensive research to elucidate the electrophysiological, biochemical, and morphological characteristics of these cells, but little attention has been paid so far to their surface membrane structure.

While seeking specific sympathetic surface markers in such neoplastic cells, we have investigated the capacity of NB to bind on their membrane, the nerve growth factor (NGF), a protein capable of inducing differentiation and maintaining the life of sympathetic cells (3). This report describes some of the properties of this interaction and introduces preliminary data on the partial characterization of a specific NGF membrane receptor site.

Materials and Methods

Protein and Chemicals.—Human serum albumin (HSA), horse spleen ferritin, and mouse and rabbit gamma globulins (MGG and RGG) were purchased from Mann Research Labs., Inc., New York; trypsin (bovine pancreas), from Worthington Biochemical Corp., Freehold, N. J.; fetal bovine serum (FBS) from Flow Laboratories, Inc., Rockville, Md.; trypticase soy broth (TSB) from Baltimore Biological Laboratories, Cockeysville, Md.; calf serum (CS) from Grand Island Biological Co., Grand Island, N. Y.; ethylenediaminetetracetate-sodium salt (EDTA) and sodium-azide from Sigma Chemical Co., St. Louis, Mo.; and ammonium sulfate from Merck Chemical Div., Merck & Co., Inc., Rahway, N. J.

Source and Preparation of Cells.—The following tumor cell lines were used: (*a*) a monocloned mouse C 1300 NB from a line kindly supplied by Dr. François Jacob, Institut Pasteur, Paris, France. The cell line was maintained in tissue culture in Dulbecco's modified Eagle's medium (DME) supplemented with 20% FBS in 12% CO_2 in air. After cloning in this laboratory, the cells were homogeneous with respect to synchronization, morphology, staining characteristics, and sedimentation. When transferred to serological petri dishes and grown in medium

[1] *Abbreviations used in this paper:* CS, calf serum; DME, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; K, relative average association constant value; MEMG, MEM plus 0.4 mg of MGG; MGG, mouse gamma globulin; NB, neuroblastoma; NGF, nerve growth factor; NSR, NGF-specific receptor-like molecule; RGG, rabbit gamma globulin.

lacking serum, most cells attached within 24 h and in 2–3 days sent out processes from the cell body, some of which were several millimeters long (2). (*b*) Mouse L-929 cells were grown in minimum essential medium (MEM) supplemented with 10% CS and 10% TSB. (*c*) Sarcoma S/180 cells were obtained from the American Type Culture Collection and maintained in MEM supplemented with nonessential amino acids and 5% CS. (*d*) Sarcoma S/37 cells were obtained from Jackson Laboratories, Bar Harbor, Maine and carried for a month by serial transplantation in female Swiss Webster mice (Jackson Laboratories). The cells were then established in our laboratory as a continuous cell line growing in suspension and named 2S/37.

Other cells used were: sympathetic cells from ganglia of neonatal Swiss Webster albino mice; spleen, kidney, and liver mouse cells freed of erythrocytes and nonviable cells by osmotic shock. For experiments all cells were washed three times in MEM without serum and maintained in this medium with or without 0.005 M EDTA throughout the experiment. Cell viability was always over 90% as determined by 0.04% erythrosin dye exclusion.

3 M KCl extraction of NB Cells.—The procedure described by Reisfield et al. (4) has been performed. 2×10^8 NB cells were suspended in 30 ml of phosphate-buffered saline (PBS) (0.05 M potassium phosphate and 0.15 M NaCl), pH 7.4, containing 3 M KCl and stirred for 16 h at 4°C. Cells were then centrifuged at 1,500 *g* for 15 min and the supernate was recentrifuged at 105,000 *g* for 120 min to remove gross aggregates and used just afterwards.

NGF.—Mouse salivary glands NGF was prepared by the method described by Bocchini and Angeletti (5). The highly purified protein, isolated as a 27,500 mol wt dimer, is composed of two identical polypeptide chains linked by noncovalent bonds (6). 1 mg of this protein dissolved in 0.01 M potassium phosphate buffer has absorbancy of 1.680 at 280 nM OD readings. The biological activity of NGF was measured by the test of Levi-Montalcini (3); 10 ng of NGF induced differentiation in vitro of 14 day chick embryo sympathetic ganglia.

Radioiodination of Proteins.—The chloramine-T method described by Greenwood et al. (7) and modified slightly by Revoltella et al. (8) was used. Radioactivity measurements were done in a LKB automatic spectrometer (Wallac Gamma Sampler Counter GTL, 300–500, LKB, Stockholm, Sweden) equipped with an NaI crystal. More than 95% of the radioiodinated proteins were precipitated by specific hyperimmune antibodies at equivalence. The counts precipitated by a known amount of ligand were considered as the "specific activity" of the ^{125}I-labeled protein expressed as cpm/weight unit. Preparations ranging between 5×10^5 to 10^6 cpm/μg protein were generally used in experiments. Iodination did not alter the biological activity of the NGF.

Binding of [^{125}I]Ligands to Cells.—A constant number of viable cells and increasing concentration of ^{125}I-labeled ligand were incubated in duplicate at 2°C in 100 × 7.5-mm disposable glass tubes. The ligand was diluted with MEM to which was added 0.4 mg MGG/ml (MEMG) to reduce nonspecific uptake by glass borosilicates. The final volume of the reaction mixture was 0.5 ml. Tubes containing the iodinated ligand and no cells were used as controls. After incubation for 30 min, tubes were centrifuged at 750 *g* for 5 min in a refrigerated International Centrifuge (International Equipment Co., Needham Heights, Mass.). The pellet was resuspended in 2.0 ml cold diluent, washed twice, and resuspended in 0.5 ml. An aliquot of this suspension was assayed for radioactivity. The counts in the controls were subtracted to determine the value for specific binding by the cells. From the specific activity of the [^{125}I]ligand preparation, the uptake of radioactivity by the cells measured at saturation could be calculated as follows:

$$\text{moles of } [^{125}\text{I}]\text{ligand bound by } 10^6 \text{ cells} = \frac{\text{cpm at the plateau of saturation}}{\text{specific activity (in cpm/mole)}}.$$

A relative average association constant value (K) was calculated from a Scatchard plot of specific binding of [^{125}I]NGF to cells, using the relationship (Bound)/(Free) = 1/K(Binding sites) − (Bound).

Binding of [^{125}I]NGF to Antibodies or Other Proteins.—The total NGF-binding capacity by antibodies or NGF specifically binding proteins was measured by a radioimmunoassay, following the procedure described by Revoltella et al. (8). In the standard assay, increasing concentrations of [^{125}I]NGF were incubated in glass tubes with 10 μg of the protein in test. After 2 h of incubation at room temperature, ammonium sulfate at a 37% final concentration was added to the reaction mixture. Tubes were centrifuged at 1,200 *g* for 30 min and the pellet washed once. The last pellet was resuspended in 0.5 ml of 0.1 N NaOH and 0.2 ml counted in the gamma spectrometer. By knowing the specific activity of the [^{125}I]NGF preparation, the moles of ligand bound by 10 μg of the substance in test was calculated from the counts at the plateau of saturation.

Acrylamide Gel Electrophoresis.—Electrophoresis of proteins dissolved in 0.2% sodium dodecyl sulphate (SDS) was performed on 7.5% acrylamide gel (6 x 80 mm, 3 mA/gel, pH 8.3 at room temperature). Gels were stained with Coomassie Blue for quantitative determination of proteins. Radioactivity was assayed by autoradiography of dried gels. The molecular weight of NGF-binding protein(s) was determined by SDS gel electrophoresis, as described by Shapiro et al. (9).

Amino Acids Analysis.—The proteins were dissolved in 0.02 N NaOH, adjusted to 6 N HCl with constant boiling HCl, and lyophilized for 24 h at 100°C in sealed evacuated tubes. Amino acid analysis was performed according to the method of Spackman et al. (10).

RESULTS

Binding of [^{125}I]NGF to Cells.—The effect of the concentration of [^{125}I]NGF on the amount of ligand bound by the cells was determined. In addition, the binding capacity of [^{125}I]NGF was compared to that of unrelated proteins such as RGG, MGG, and ferritin. The number of moles of [^{125}I]NGF bound by NB cells at every ligand concentration was 20–30 times higher than the number of moles of the other ligands tested (Fig. 1).

Specificity of Binding of [^{125}I]NGF by NB.—In order to determine whether NGF bound preferentially to NB cells, binding was compared in a series of normal and tumor cells (Fig. 2). The ratios of [^{125}I]NGF bound by NB cells and by equal numbers of other cells were calculated at saturation of the ligand-binding capacity. NB cells and sympathetic cells showed a much greater

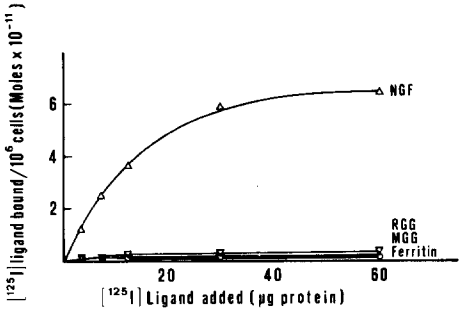

FIG. 1. Binding of different [^{125}I]ligands to a constant number of C 1300 NB cells. Mol wt of NGF and ferritin were taken, respectively, as 27,500 and 700,000; of MGG and RGG, as 150,000.

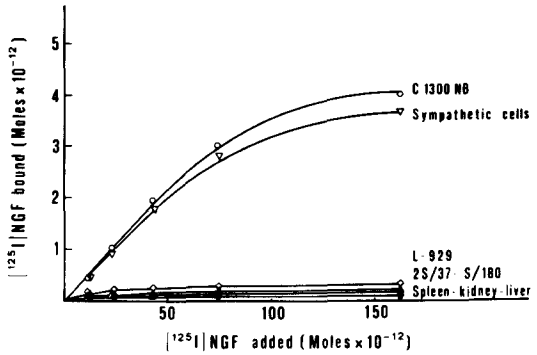

FIG. 2. Binding of [^{125}I]NGF to cells. Similar number of C 1300 NB, L-929, 2S/37, S/180, and normal mouse sympathetic cells were incubated for 30 min at 2°C with increasing concentrations of the ligand. Amount of cell-bound [^{125}I]NGF was determined after two washes with 2 ml of MEMG.

capacity for binding [^{125}I]NGF than other cells, even at low ligand concentrations. These differences were even more striking when corrections were made for differences in the various cells. From measurements of the diameters of 20–30 cells of each type, it was determined that NB cells (ca. 40 μm diameter) are intermediate in size between L-929 and 2S/37 or S/180 (respectively 70, 40, and 35 μm diameter) but are much larger than normal mouse spleen leukocytes, or kidney or liver cells, which are much more similar in size to sympathetic cells. If each cell is considered as a sphera and the amount of binding is expressed in terms of the amount per unit area, preferential binding by NB or sympathetic cells was still evident, thus excluding the possibility that the differences can be explained on the basis of cell size. The binding of [^{125}I]-NGF by increasing numbers of NB cells is shown in Fig. 3. Values of [^{125}I]NGF bound are expressed as specific binding by the cells, i.e. moles of ligand bound to NB cells minus the average number of moles of ligand bound by equal numbers of L-929, 2S/37, and S/180 control cells. The average binding of [^{125}I]-NGF by these latter cell controls was not significantly different from the number of moles of the same ligand bound by the globulins in the medium diluent. As can be seen, there was a direct proportion of binding in relation to cell number.

Effect of Different Factors on the Binding of [^{125}I]NGF to Cells.—It was determined that saturation of binding occurred within 15 min and a standard incubation time of 30 min was adopted. Repeated washing with MEMG removed only about 50% of the [^{125}I]NGF bound to NB cells. In contrast, washing removed almost all the [^{125}I]NGF from control cells [Fig. 4). The effect was greatest with the first wash, whereas the next wash affected only the controls. Therefore, two washes were used in the standard assay procedure.

The effect of prefixation with glutaraldehyde on binding of [^{125}I]NGF by NB

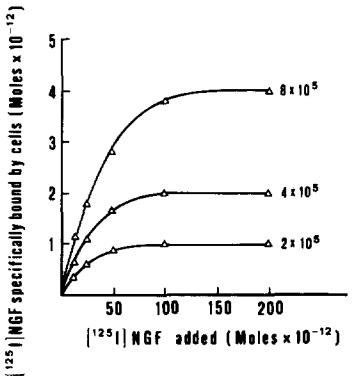

FIG. 3. Specific binding of [^{125}I]NGF as a function of C 1300 NB-cell concentration. Binding by L-929 cells was subtracted to yield values of specific binding.

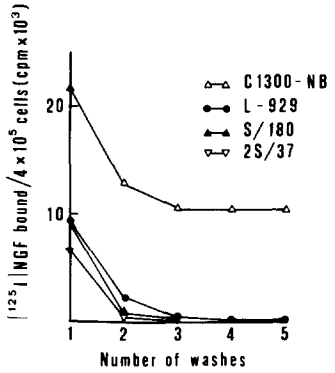

FIG. 4. Effect of washing on binding of [^{125}I]NGF. Similar number of C 1300 NB and L-929, 2S/37, or S/180 cells were incubated for 30 min at 2°C. The quantity of ligand remaining bound to cells was measured after increasing washes with 2 ml of MEMG.

and L-929 cells is shown in Table I. A significant increase in binding was shown by both types of cells, in particular the control cells. This may be explained by increased availability after fixation of low avidity binding sites (11–13).

Binding of NB cells was greatly reduced shortly after the cells were treated with 0.02% trypsin. However, binding capacity was recovered and after 3 h the same amount of NGF was bound as before treatment, indicating rapid replacement on the cell membrane of NGF-binding molecules.

Divalent cations had no effect on binding reaction. When 2×10^5 NB cells were incubated with the ligand, 3.1×10^{-12} mol of [^{125}I]NGF were bound at saturation. In the presence of 0.005 or 0.01 M EDTA the same number of cells bound 2.78×10^{-12} and 3.21×10^{-12} mol of [^{125}I]NGF, respectively.

Identity on Cell-Binding Capacity between Labeled and Unlabeled NGF.—When

TABLE I

Effect of Different Treatments on Binding of [^{125}I]NGF by Cells at Saturation

Cells	No treatment	Trypsin*			Glutaraldehyde‡
		10 min	60 min	180 min	
	cpm	*cpm*	*cpm*	*cpm*	*cpm*
C 1300 NB	32,518	1,214	18,318	35,141	275,418
L-929	1,321	818	1,312	1,280	25,421

* 2×10^7 cells were incubated for 10 min at 37°C with 0.02% trypsin. Cells were washed three times and then incubated after 10, 60, and 180 min with [^{125}I]NGF at 2°C. Cell viability was higher than 85–90%.

‡ 2×10^7 cells were incubated in 1% glutaraldehyde in MEM for 10 min and then thoroughly washed with medium supplemented with 0.02 M glycine.

a decreasing number of NB cells was incubated with a constant amount of [^{125}I]NGF, binding curves such as the upper one in Fig. 5 were obtained. The sigmoid curve leads to two plateaus, the upper representing a condition of complete saturation of the [^{125}I]NGF by excess-binding sites, the lower indicating an excess of ligand as compared to available receptor sites. When unlabeled and ^{125}I-labeled NGF were added together to the reaction tubes before adding cells, modifications of the binding curve occurred, indicating that unlabeled NGF competitively bound to cells and prevented fixation of [^{125}I]NGF to some cell receptors. The binding capacity at different cell concentrations of NGF of the four different resulting specific activities, was then analyzed by plotting values in terms of the reciprocal of bound vs. the reciprocal of free [^{125}I]NGF (8). At a cell concentration corresponding to about 30% of the maximal cell-binding capacity for [^{125}I]NGF (8×10^4 cells) a straight line can be drawn, as shown in the inset of Fig. 5. Curves bent towards the ordinate or the abscissa were drawn, respectively, at smaller or higher cell concentrations. The straight line therefore represents a situation of equilibrium between different avidity receptor sites (8). The intercept in the ordinate of this straight line represents the reciprocal value of the total [^{125}I]NGF moles bound by that number of cells. This value closely corresponds to the same concentration of [^{125}I]NGF bound, which can be calculated at saturation from a radioimmunoassay on the same cells. This shows that even if there is heterogeneity of binding capacity by the cells, there is identity between labeled and unlabeled NGF.

K and Number of Binding Sites per Cell.—K for the [^{125}I]NGF interaction with NB cells was calculated from a curve of specific binding at different ligand concentrations, up to a plateau of saturation (Fig. 6), using the relationship (Bound)/(Free) = 1/K (Binding sites) − (Bound) (14). In several independent experiments the values for K ranged between 0.8 and 5.6×10^7 liters/mol. 10^6 NB cells bound on an average 6×10^{-12} mol of [^{125}I]NGF (Fig. 6). By the Avogadro number, this figure corresponds to about 10^6 molecules of [^{125}I]NGF bound/cell. NGF is, however, a multivalent ligand and each

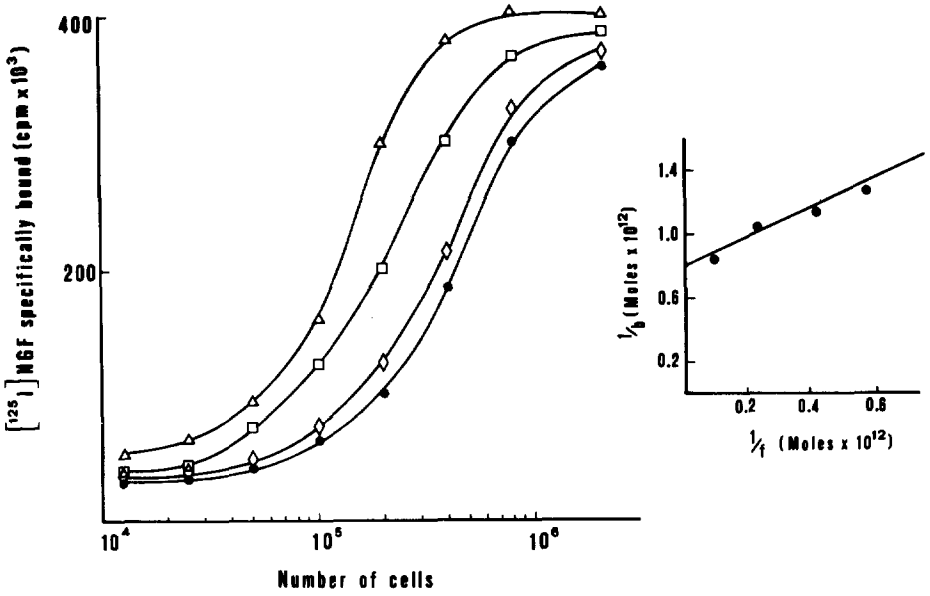

FIG. 5. Inhibition of [^{125}I]NGF uptake by unlabeled NGF. 2.5×10^{-12} moles of [^{125}I]NGF (sp act, 160,000 cpm/10^{-12} mol) were added to all tubes. △—△, no unlabeled NGF added; □—□, 0.8×10^{-12} mol of unlabeled NGF added; ◇—◇, 2.5×10^{-12} mol of unlabeled NGF added; and ●—●, 7.5×10^{-12} mol of unlabeled NGF added. The Sips plot of data at a cell concentration of 8×10^4 allowed the calculation of the total NGF-binding capacity by cells which were 1.31×10^{-12} mol. By a direct immunoassay performed on cells of the same source, the recovery was 1.8×10^{-12} mol of NGF bound/8×10^4 cells.

molecule could independently interact with more than one receptor site on the cell surface. It is therefore arbitrary to calculate an exact number of binding sites from this data. Only a minimum value can be calculated with the assumption that the number of binding sites available on the cell surface is equal to or greater than the number of [^{125}I]NGF molecules bound.

Preliminary experiments suggested that exposure of NGF-binding sites on the NB-cell membrane might vary with the cell cycle and therefore binding experiments were done on synchronized cells. NB synchronization was achieved by culturing the cells for 48 h in DME lacking isoleucin at pH 6.5 (15–16). Maximal exposure of binding sites for NGF occurred during a restricted phase of the cycle which preceeds DNA synthesis ([^{3}H]thymidine incorporation) (Table II). At the same time, the K value of the interaction changed during different stages of the cell cycle, with maximal values obtained at stages of maximal NGF-binding capacity.

Displacement of Bound [^{125}I]NGF by Unlabeled NGF, In Relation to NB Growth Cycle.—Increasing concentrations of unlabeled NGF (from 10^{-3} to 10^{+2} μg of protein) were added to tubes containing 5×10^5 cells and 0.05 μg protein of

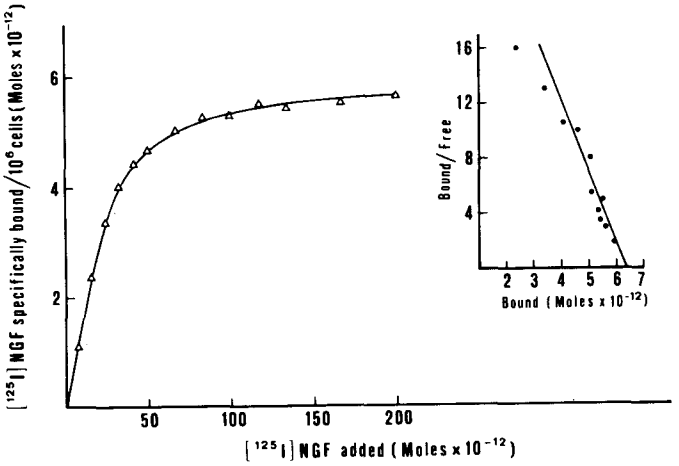

FIG. 6. Binding as a function of concentration of [^{125}I]NGF, and Scatchard plot of these data. On the Scatchard plot, the slope is equivalent to 1/K.

TABLE II

Binding of [^{125}I]NGF by Synchronized C 1300 NB Cells During a Growth Cycle

Time	[^{3}H]Thymidine uptake*	[^{125}I]NGF bound/10^6 cells‡	K
h	*cpm/10^6 cells*	*moles × 10^{-12}*	*10^7 liters/mol*
0	300	34.00	4.60
2	370	42.00	6.20
4	1,105	1.00	2.00
6	14,026	0.02	0.80
8	4,378	0.08	0.93
10	1,712	0.10	1.31
12	419	0.70	2.56

* [^{3}H]Thymidine sp act 5 Ci/mmol, dosage 0.5 μCi/ml, pulse time 1 h.

‡ [^{125}I]NGF, sp act 1.7 × 10^6 cpm/μg protein.

intensively iodinated NGF (sp act 1.5 × 10^6 cpm/μg). The number of counts bound decreased proportionately. When NB cells synchronized in the G_1 phase were used, complete displacement of labeled NGF required 50 μg protein of unlabeled NGF, whereas with cells in the late S phase, this required only 0.4 μg of protein (Fig. 7).

Partial Characterization of NGF-Specific Receptor (NSR).—When [^{125}I]NGF was added dropwise at 37°C to the 105,000 *g* supernate obtained after 3 M KCl extraction of NB cells, precipitate appeared almost instantly. This pellet was washed three times and dissolved in SDS. Acrylamide gel electrophoresis showed that after removal of the pellet the supernate was lacking one major protein component, representing about 2–5% of the total extracted proteins.

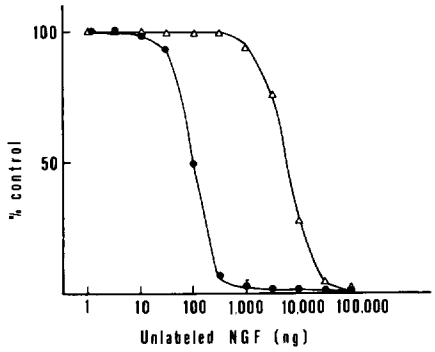

FIG. 7. Displacement of binding of 0.05 μg of [^{125}I]NGF from 5 $\times$ 10^5 C 1300 NB cells, by increasing concentrations of unlabeled NGF, is considered as 100%. Cells were synchronized in the G_1 phase (▲—▲) or in the late S phase (△—△) of the growth cycle.

(Fig. 8). Molecular weight determinations on SDS acrylamide gels, show that the purified material (NGF-specific-receptor-like molecule, tentatively called NSR) has a mol wt of about 52,000. The amino acid composition of NSR obtained from the gel band (Table III) indicates a rather high concentration of aspartic and glutamic acids, a composition very similar to that obtained with tubulin proteins (17). Very similar results from different purified NSR preparations were obtained, yielding almost identical amino acid patterns, suggesting high purity and specificity of the material. Large amounts of NSR could be isolated after paper electrophoresis. The direct interaction between highly purified NSR and [^{125}I]NGF was then measured by a radioimmunoassay at 2°C. At this low temperature, before adding the ammonium sulfate salt, no spontaneous precipitate was obtained, at least after a few hours from the moment of the interaction of the reagents. In the radioimmunoassay, a saturable-binding curve was obtained, the process following Michaelis-Menten kinetics. From a Scatchard plot of the data of specific binding (e.g., total binding subtracted from the nonspecific binding due to the diluent) K in the range of 10^7 literes/mol was obtained.

Inhibition of NGF Binding by NB Cells Incubated with Anti-NSR Antibody.— NB cells were suspended in DME and incubated with heat-decomplemented rabbit antibodies prepared against highly purified NSR. As controls, cells were incubated with rabbit antiferritin antibodies or normal RGG, all diluted to the same final protein concentration. Each of the globulin fractions was first absorbed at 4°C for 16 h with a suspension of kidney cells from A/J mice, the same strain from which the C 1300 NB cells originated. After incubation with antibody, cells were washed twice and exposed to [^{125}I]NGF. The binding capacity of 10^6 cells incubated with normal gamma globulin or antiferritin antibodies was 5.72 $\times$ 10^{-12} and 6.18 $\times$ 10^{-12} mol of [^{125}I]NGF, respectively.

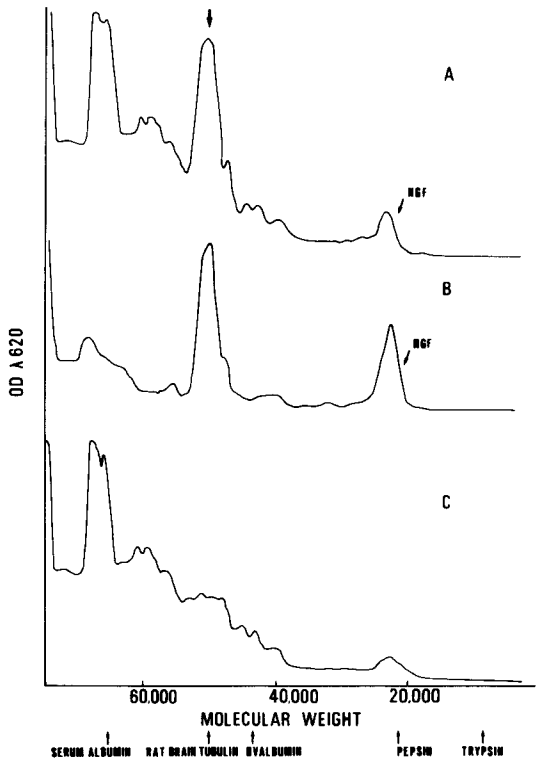

FIG. 8. Electrophoretic patterns of NB proteins extracted by 3 M KCl and incubated in vitro with NGF. Densitometer tracing of: (A), total soluble cell extract and NGF; (B), pellet obtained by centrifugation at 105,000 *g* for 1 h, after incubation at 37°C for 60 min in the presence of 10^{-3} mM $MgCl_2$; and (C), supernate obtained after the above reaction. Arrow indicates the position of the protein specifically binding NGF (NSR).

Anti-NSR antibodies reduced the binding capacity to less than 6.2×10^{-14} mol/10^6 cells.

Cytotoxic Effect of Anti-NSR Antibodies on NB Cells.—A specific cytotoxic effect of rabbit anti-NSR or antipurified rat brain tubulin antibodies was obtained when NB cells were incubated at 37°C for 60 min in the presence of complement (guinea pig serum diluted 1/5). Antiferritin antibodies and normal gamma globulins were not cytotoxic, at the same protein concentrations (Table IV). When synchronized cells were used, the cytotoxic effect after incubation at 37°C for 30 min was much more pronounced in cells at a phase of the cell cycle immediately preceeding DNA synthesis (Table IV).

DISCUSSION

The evidence presented in these experiments clearly indicates that murine C 1300 NB and normal sympathetic cells bind NGF in vitro, at 2°C, on their

TABLE III

Amino Acid Composition of (NGF) Binding Protein(s)

Amino acid	Tubulin*	NGF-precipitated proteins	
		Mouse brain extract	C 1300 NB extract
		mole percentage	
Lysine	6.51	6.1	6.21
Histidine	2.74	2.0	2.35
Arginine	5.62	5.2	5.74
Aspartic acid	10.24	10.0	10.51
Threonine‡	6.41	6.5	6.18
Serine‡	5.62	7.0	5.54
Glutamic acid	13.18	13.0	13.41
Proline	5.02	5.1	5.04
Glycine	8.14	—	8.18
Alanine	8.08	8.7	7.64
Half cystine	1.68	—	1.61
Valine	6.51	6.8	6.24
Methionine	2.74	—	3.18
Isoleucine	4.98	4.4	4.81
Leucine	8.50	7.15	7.52
Tyrosine	3.02	—	3.38
Phenylalanine	4.12	3.8	4.34

Mole percentage for tubulins. Average of duplicate sets of 24-h hydrolysis.

* Purified tubulin prepared as described by Shelanski et al. (28). The NGF-precipitated proteins were obtained by addition of NGF at 37°C to a 105,000 *g* supernate of mouse brain homogenate prepared as described in the test. The NGF-precipitated proteins from murine C 1300 NB proteins were obtained by addition of NGF to a 105,000 *g* supernate of 3 M KCl extract, prepared as described by Reisfield et al. (4).

‡ Threonine and serine were determined by extrapolation to zero hydrolysis time.

membrane surface. These results are in line with recent observations by Banerjee et al. (18) on human sympathetic cells. The results of the experiments can be summarized as follows: (*a*) the binding of [^{125}I]NGF is not mediated by divalent cations, since 0.01 M EDTA had no inhibitory effect; (*b*) the binding is not dependent on the cell metabolic activity, as indicated by the fact that it takes place at 2°C or in presence of Na-azide, or after cell fixation in glutaraldehyde; (*c*) the receptor for NGF binding is most likely of a protein nature, as suggested by the fact that trypsin abolished the binding capacity by the cells, however the binding reappears within 1–2 h, suggesting that such receptors might play a significant role in the cell membrane function; (*d*) the binding is specific for NGF in view of the fact that it can be inhibited by unlabeled NGF. In addition, NB cells exhibited a [^{125}I]NGF-binding capacity several times higher than the binding exhibited by a variety of other ligands tested (Fig. 1). As can be seen in the figure, at saturation of binding capacity, 10^6 NB cells bound about 6×10^{-12} mol of [^{125}I]NGF, a value which is 70–80

TABLE IV

*Cytotoxic Effect of Rabbit Antibodies on Synchronized Murine C 1300 NB Cells In Vitro**

Time	[^{3}H]Thymidine uptake‡	Cytotoxic effects			
		Normal§ gamma globulins	Antibodies§		
			Antiferritin	Anti-NSR	Antirat brain tubulin
h	*cpm/10^6 cells*	*% viability*			
0	254	95	97	25	24
3	318	91	91	12	5
6	18,217	92	93	51	62
9	2,351	91	92	64	71
12	541	92	94	71	74
15	618	94	95	70	78

* Cells were incubated for 60 min at 37°C, in presence of added complement (guinea pig serum diluted 1/5), with normal RGG or different types of antibodies. Viability of cells was scored by 0.04% erythrosin dye exclusion.

‡ [^{3}H]thymidine, sp act, 5 Ci/mM; dosage, 0.5 μCi/ml; pulse time, 1 h.

§ Globulin fractions obtained from sera precipitated at 35% ammonium sulfate saturation. Sera had been previously absorbed at 4°C for 16 h with a suspension of kidney cells from A/J mice. Rabbit anti-NSR and antirat brain tubulin antibodies had, respectively, 42 μg and 38 μg antibody protein/ml as determined by a quantitative precipitin test using purified tubulin as antigen. All globulins were diluted to the same final protein concentration before exposure to cells.

times higher than the average binding capacity exhibited for other tested ligands. Moreover, the binding capacity exhibited at saturation by NB cells or sympathetic mouse cells (Fig. 2) was 40–70 times higher than that shown by an equal number of other tumor or normal cells. Such a preferential expression of sites capable of specifically binding the NGF molecule probably justifies the in vitro and in vivo well-documented unique responsiveness to NGF by these target cells (3). From our data it would result that about 10^5–10^6 molecules of [^{125}I]NGF can be bound at saturation by a single NB cell in suspension at 2°C, exhibiting a K in the range of 10^7 liters/mol. This number of bound molecules may be even higher, since indentation, folding, and refolding of the cell membrane may mask receptor sites. This number fluctuates, however, during the different phases of the cell cycle and so does the average value of the relative association constant of their interaction.

This phenomenon was already described in previous reports, using a technique of rosette formation by NB cells incubated at 2°C for a short period of time, with sheep erythrocytes passively coated with NGF.[2] In agreement with these preliminary observations, the results presented in Table II indicate that

[2] Revoltella, R., C. Bosman, and L. Bertolini. 1974. Nerve growth factor binding sites in synchronized murine C 1300 neuroblastoma-rosette forming cells. Specificity and morphology of rosette formation. *Cancer Res.* Manuscript submitted for publication.

the binding capacity by NB cells was maximal during the late G_1 and early S phase. Displacement experiments reported in Fig. 7 gave additional confirmation to these findings, indicating different requirements of cold NGF to displace a certain amount of labeled NGF from the membrane surface of synchronized NB cells, at different phases of their cycle. These findings may be justified in view either of a possible different expression of receptor molecules. onto the membranes, or to rearrangements of these molecules, in relation to the physiological requirements of the cell during the different phases of the cycle.

In both cases a variation in cell-binding capacity occurs. Restricted expression of receptors during the cell cycle has been also demonstrated in many different systems, in bacteria (19) or mammalian cells (20–22), and probably represents a critical step in the life controlling their further proliferation and/or differentiation (23); (*e*) the question is now raised as to the nature of this NGF-specific-binding protein. Preliminary experiments have been performed with the aim of isolating from the NGF target cells such molecules. A protein(s), of about 52,000 mol wt (exhibiting a K with NGF of about 10^7 liters/mol), could be isolated from the supernate of NB cells after their exposure for several hours, in the cold, to 3 M KCl. The amino acid composition of such purified NGF specifically binding material (Table III) revealed a remarkable similarity with a protein which is known to be a fundamental component of microtubules, named tubulin (17). The idea of considering tubulin as a possible receptor for NGF was supported by recent investigations in our laboratory by Calissano et al. (24) who showed that purified rat brain tubulin exhibits in vitro a K of binding for monomeric NGF in the range of 10^6–10^7 liters/mole. A possible identity ot the protein responsible for NGF binding to its target cell and tubulin came also from experiments performed with antibodies against NSR or a purified preparation of tubulin obtained from rat brains. These experiments showed that these antibodies, when added to a suspension of NB cells, inhibited the binding of NGF to the cell membrane. In the presence of the complement these antibodies produced a specific cytotoxic effect on the target cells which was maximal in correspondence to the G_1 phase of the cycle, at their NGF higher-binding capacity. Antibodies made against ferritin or normal gamma globulin had no effect (Table IV).

It is also known that microtubule proteins are rather obiquitous in all cellular systems so far investigated, and chemically or immunologically almost indistinguishable (25). It is tempting to correlate the similarity of these findings with the possibility that insoluble microtubule proteins, exposed onto the membrane of NB or sympathetic cells, might by themselves act as a source of receptor sites for NGF. At variance with this hypothesis, these microtubule proteins might cooperate with membrane tightly associated specific NGF receptors, undergoing polymerization and tubule formation after the receptors interaction with NGF molecules, as suggested in a different experimental model by Edelman et al. (26). In normal sympathetic cells, such a reaction probably might represent the initial step of neuron differentiation (27).

SUMMARY

Murine C 1300 neuroblastoma cells bind with high avidity on their membrane surface the nerve growth factor (NGF), a protein capable of inducing differentiation of sympathetic nerve cells. The total binding capacity of NGF by the cells was quantitatively measured by a radioimmunoassay technique, using ^{125}I-labeled NGF. An average number of about 10^6 molecules of NGF could be bound, at saturation, by each cell with an average relative association constant of about 10^7 liters/mol. Using synchronized cells, it was found, however, that either the number of molecules of ligand bound or the avidity of the binding interaction between NGF and cells varied depending upon their growth cycle, the maximal-binding occurring during the G_1 and early S phase. Binding of [^{125}I]NGF was suppressed by trypsin treatment of the cells, however new receptor sites were rapidly replaced onto the membrane surface within 1–2 h. Cells exposed to 3 M KCl released into the supernate a protein product exhibiting similar high avidity for NGF. Acrylamide gel electrophoresis suggested a restricted molecular heterogeneity of this product, with a major component in the 52,000 mol wt region. Antibodies made specific to this protein were capable, in the absence of the complement, of inhibiting the binding of [^{125}I]NGF by the cells and in the presence of the complement they killed them.

REFERENCES

1. Schubert, D., S. Humpreys, C. Baroni, and M. Cohn. 1969. In vitro differentiation of a mouse neuroblastoma. *Proc. Natl. Acad. Sci. U. S. A.* **64**:316.
2. Schubert, D., S. Humpreys, F. de Vitry, and F. Jacob. 1971. Induced differentiation of a neuroblastoma. *Dev. Biol.* **25**:514.
3. Levi-Montalcini, R., and P. U. Angeletti. 1968. Nerve growth factor. *Physiol. Rev.* **48**:534.
4. Reisfield, R. A., M. A. Pellegrino, and B. D. Kahan. 1971. Salt extraction of soluble HL-A antigens. *Science (Wash. D. C.)*. **172**:1134.
5. Bocchini, V., and P. U. Angeletti. 1968. The nerve growth factor: purification as a 30,000 molecular weight protein. *Proc. Natl. Acad. Sci. U. S. A.* **64**:787.
6. Angeletti, R. H., R. A. Bradshaw, and R. C. Wade. 1971. Subunit structure and amino acid composition of mouse submaxillary gland nerve growth factor. *Biochemistry*. **10**:463.
7. Greenwood, F. C., W. M. Hunter, and J. J. Glover. 1963. The preparation of [^{131}I]-labeled human growth hormone of high specific radioactivity. *Biochem. J.* **89**:114.
8. Revoltella, R., R. H. Angeletti, M. Pediconi, and L. Bertolini. 1974. Radioimmunoassay for the measurement of mass and avidity of anti-nerve growth factor antibodies. *J. Immunol. Meth.* **4**:67.
9. Shapiro, A., E. Vinuela, and J. V. Maizel, Jr. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrilamide gels. *Biochem. Biophys. Res. Commun.* **28**:815.
10. Spackman, D., S. Moore, and M. Stein. 1958. Automatic recording apparatus of use in the chromatography of amino acids. *Anal. Chem.* **30**:1190.

11. Marchalonis, J. J., R. E. Love, and J. L. Atwell. 1972. Isolation and partial characterization of lymphocytes surface immunoglobulins. *J. Exp. Med.* **135**:956.
12. Haskill, J. S., P. E. Elliott, R. Kerbel, M. A. Axelrad, and D. Eidinger. 1972. Classification of thymus-derived and marrow-derived lymphocytes by demonstration of their antigen-binding characteristics. *J. Exp. Med.* **135**:1410.
13. Revoltella, R., G. Martinelli, and A. G. Osler: 1972. The antigen-binding capacity of mouse lymphocytes. Changes during the early immune response. *Cell. Immunol.* **6**:215.
14. Yung Lee, C., and R. J. Ryan. 1972. Luteinizing hormone receptors: specific binding of human luteinizing hormone to homogenates of luteinized rat ovaries. *Proc. Natl. Acad. Sci. U. S. A.* **69**:3250.
15. Tobey, R. A., and K. D. Ley. 1971. Isoleucine-mediated regulation of genome replication in various mammalian cell lines. *Cancer Res.* **31**:46.
16. Revoltella, R., L. Bertolini, and M. Pediconi. 1974. Unmasking of nerve growth factor membrane specific binding sites in synchronized murine C 1300 neuroblastoma cells. *Exp. Cell Res.* **85**:89.
17. Olmsted, S. B., K. Carlson, R. Klebe, F. Ruddle, and J. Rosenbaum. 1970. Isolation of microtubule protein from cultured mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **65**:129.
18. Banerjee, S. P., S. H. Snyder, P. Cuatrecasas, and L. A. Greene. 1973. Binding of NGF receptor in sympathetic ganglia. *Proc. Natl. Acad. Sci. U. S. A.* **70**:2519.
19. Sueoka, N. *In* Cell Synchrony Studies in Biosynthetic Regulation. I. L. Cameron and G. M. Padilla, editors. Academic Press, Inc., New York. 38.
20. Peterson, D. F., R. A. Tobey, and E. C. Anderson. 1968. Developmental biology of normal cells: biology and biochemical aspects. *Cancer Res.* **28**:1821.
21. Buell, D. N., and J. L. Fahey. 1969. Limited periods of gene expression in immunoglobulin synthesizing cells. *Science* (*Wash. D. C.*). **164**:1524.
22. Byers, N., and C. Kidson. 1970. Programmed synthesis and export of immunoglobulins by synchronized myeloma cells. *Nature* (*Lond.*). **226**:648.
23. Prescott, D. M. 1968. Regulation of cell reproduction. *Cancer Res.* **28**:1815.
24. Calissano, P., and C. Cozzari. 1973. Interaction of NGF with the mouse brain neurotubule protein(s). *Proc. Natl. Acad. Sci. U. S. A.* In press.
25. Dales, S. 1972. Concerning the universality of a microtubule antigen in animal cells. *J. Cell. Biol.* **52**:748.
26. Edelman, G. R., I. Yahara, and J. L. Wang. 1971. Receptor mobility and receptor-cytoplasmic interactions in lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* **70**:1142.
27. Yamada, K. M., AND N. K. Wessel. 1971. Axon elongation. Effect of nerve growth factor on microtubule protein. *Exp. Cell Res.* **66**:346.
28. Shelanski, M. L., F. Gaskin, and G. R. Cantor. 1973. Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. U. S. A.* **70**:765.