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A Serological Assay for the Detection of Cell Surface Receptors of Nerve Growth Factor

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When single-cell suspensions prepared from embryonic day 8 (E8) chick sensory ganglia are incubated with nerve growth factor (NGF), anti-NGF antiserum, and complement, an NGF-dependent cytotoxic kill of 20 (±3)% of the ganglia cells is observed. This percentage is increased by a factor of two when only the neuronal cells are tested. No kill is observed on the nonneuronal cell population representing 50% of the ganglia dissociate. When E8 sensory ganglia cells are cultured in the presence of NGF following cytotoxic kill, the large, phase-bright NGF-reponsive neurons are missing from the culture. These results indicate that the cells recognized in the cytotoxicity assay have to carry NGF-binding sites of type I, which is the one with the higher affinity of the two types of NGF-binding sites (I and II) present on sensory ganglia cells. This conclusion is further supported by the following data: a) half maximal cytotoxicity is reached already at a concentration of NGF which is below the K_D of binding site I; b) a washing step which removes all NGF bound to type II receptors while leaving a high percentage of type I receptors occupied has no effect on the percentage of ganglia cells killed.

Using the cytotoxicity assay the presence of high-affinity binding sites of type I can be demonstrated on sensory ganglia cells from E8 chick embryos but not from E4 embryos and not on liver and heart cells from E8 embryos. Further, type I receptor-bearing cells were detectable in the brain using this assay. At E8, NGF receptors could be detected on cells of the forebrain and the tectum but not on brain stem cells. Cytotoxic kill of forebrain cells was found to be especially high at E8 and E9, and decreased by E10.

Key words: nerve growth factor, receptors, sensory ganglia cells, brain cells, serological receptor assay

Nerve growth factor (NGF) is a polypeptide which plays a crucial role in the development and maintenance of sensory and sympathetic neurons in vivo as well as in vitro [1]. In binding studies with radiolabeled NGF we have shown that at certain stages of embryonic

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development there are two distinct classes of high-affinity binding sites (I and II) for NGF on sensory ganglia cells of the chick. One of the two (site I) was found to be responsible for the NGF-dependent neurite extension of sensory neurons in vitro [2, 3]. Because of disadvantages of the binding assays with ^{125}I-NGF, – the radiolabeled NGF preparation can only be used within the relatively short time span of three weeks, each preparation has to be checked for its molecular homogeneity, and the binding assays require a large number of cells – we developed an alternative assay to screen for the presence of NGF receptors during embryonic development. This communication describes a serological test – an antiserum- and complement-mediated cytotoxicity assay – as a fast, easy, and sensitive assay procedure for the detection of NGF receptor-bearing cells. The principle of the assay described has been used for a long time by immunologists to test for the presence of certain cell surface antigens on cells of the lymphoid system, thereby defining and characterizing various lymphocyte subclasses [4]. The NGF-dependent cytotoxicity assay deviates from the classic procedure insofar as the antigen is not a cell surface component but a diffusible factor. The binding of this factor to specific sites on the cell surface allows the detection of the cells which carry the binding sites. We will show in the following that NGF has to bind to site I in order to mediate the cytotoxic effects of anti-NGF antiserum and complement and that binding to site II does not contribute to the NGF-mediated cytotoxic kill.

MATERIALS AND METHODS

Preparation of Materials

Nerve growth factor. NGF was purified in the form of β-NGF from the submaxillary glands of male Swiss Webster mice as described previously [5].

Preparation of single-cell suspensions. Single-cell suspensions were prepared from sensory ganglia, brain, liver, and heart using a mild trypsinization procedure. The method has been described previously [6]. Cell viability judged by Trypan Blue exclusion was $\geqslant 95\%$.

Sera. Anti-NGF antiserum was prepared in young female New Zealand rabbits as described elsewhere [7]. The antisera were used in the cytotoxixity assay following absorption with E8 chick homogenate to remove non-NGF-mediated cytotoxicity. The cytotoxic titer of the antiserum (antiserum dilution at which 50% of maximal kill occurs) varied between 1:8 and 1:64 depending on the batch of serum used. Guinea pig serum (Gibco) was used as a complement source at a dilution of 1:4.

Serological Procedures

Cytotoxicity assay. For the NGF-dependent cytotoxicity assay cells at a concentration of 6×10^6/ml were preincubated with NGF at a concentration three times higher than the final concentration chosen for the test. Preincubation time was 30 min. The incubation medium was M 199 (International Scientific Industries) containing 10% heat-inactivated, γ-globulin-free fetal calf serum (Gibco). For the cytotoxicity test 0.025 ml of this cell suspension was incubated with 0.025 ml of anti-NGF antiserum and 0.025 ml of complement diluted with the incubation medium for 30 min at 37°. Cell death was determined by uptake of Trypan Blue dye under the microscope. No further increase in cytotoxic kill was observed when cells were incubated up to 2 h with anti-NGF antiserum and complement.

Absorptions. Anti-NGF antiserum diluted 1:2 with Medium 199 containing 10% γ-globulin free heat-inactivated fetal calf serum was absorbed for 30 min with washed

homogenates of E8 chick embryos from which brain, spinal cord, and bones had been removed. The homogenate was prepared using a loose-fitting glass-Teflon homogenizer in Earle's balanced salt solution (EBSS) (International Scientific Industries). The washing procedure consisted of three washes in the same salt solution centrifuging at 250g. Then the homogenate was resuspended in Medium 199 plus 2% γ-globulin-free, heat-inactivated fetal calf serum and spun down for absorption at 500g. The ratio of antiserum to packed tissue homogenate was 5:1. All absorptions were carried out on ice.

Cell culture. Sensory ganglia cells were cultured in 35-mm plastic tissue culture dishes (Falcon) coated with poly-L-lysine (Sigma), as described elsewhere [3].

RESULTS

When anti-NGF antiserum and complement are added to a single-cell suspension prepared from sensory ganglia of 8-day-old (E8) chick embryos which has been preincubated with NGF (40 ng/ml), cytotoxic kill of 20 (± 3)% of the cells is observed. The serum concentration dependence of the cytotoxic kill is shown in Fig. 1, which also demonstrates that no kill above the serum and complement controls is observed in the absence of NGF. The partial kill of only 20% of the ganglia cells was interesting and suggested that possibly only one of the two high-affinity NGF-binding sites present on sensory ganglia cells was recognized in the cytotoxicity assay. It is known from binding assays with ^{125}I-NGF that at least part of the neuronal cells carry both site I and site II on the cell surface while the nonneuronal cells have only site II, the site with the lower affinity. At 40 ng/ml, the NGF concentration used for the assay shown in Fig. 1, all type I receptors and nearly 50% of

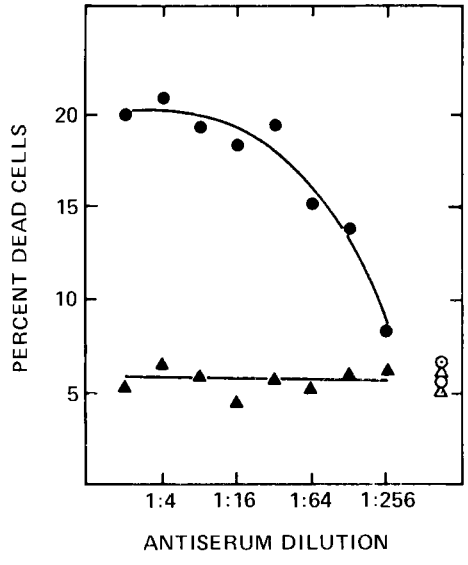

Fig. 1. Serum concentration dependence of NGF-mediated cytotoxicity. E8 sensory ganglia cells were incubated with 120 ng/ml NGF, then introduced into the cytotoxicity test as described in Materials and Methods. Final NGF concentration in the test was 40 ng/ml. Cytoxicity at 40 ng/ml NGF (●), cytotoxicity without NGF (▲), serum control (antiserum without complement) (○ △), complement control (complement without antiserum) (○ △).

the type II receptors are occupied [2]. Since much less than 50% of the cells are killed, it is likely that only site I is recognized in this assay. This is further supported by the NGF concentration dependence of the cytotoxic kill which is shown in Fig. 2. Maximal cytotoxicity is already reached at 1 ng/ml and no increase is observed up to concentrations of 100 ng/ml. Approximately 70% of sites I are occupied at a concentration of 1 ng/ml NGF; the occupancy of site II increases from 2% at 1 ng/ml to 70% at 100 ng/ml without any further increase in cytotoxicity.

The data shown in Fig. 3. further support the notion that NGF binding to site II does not contribute to the cytotoxic kill. In these experiments the cells were washed following the incubation with NGF before they were introduced into the cytotoxicity assay. From binding assays with ^{125}I-NGF the half-life of the NGF-receptor complex has been determined as 10 min for site I and 3 sec for site II [2, 3]. By means of the washing step after preincubation of the cells with NGF the free NGF and the NGF bound to site II can be removed completely, while a high percentage of sites I remain occupied. As seen in Fig. 3 the percentage of cells killed in the cytotoxicity assay is not affected by the washing step.

Sensory ganglia cell suspensions prepared from E8 chick embryos are composed of approximately equal amounts of neuronal and nonneuronal cells. The neurons can be separated from the nonneuronal cells by means of their differential adsorption to the surface of tissue culture dishes [3]. The efficiency fo the method is demonstrated by Fig. 4, which shows 24-h cultures of the ganglia dissociate and its neuronal and nonneuronal cell populations grown in the presence of NGF. The cross-contamination of the neuronal population with nonneuronal cells and vice versa can be kept below 5%. Binding assays with ^{125}I-NGF showed a doubling in the amount of ^{125}I-NGF bound to site I when the neuronal cells of the ganglia were tested compared to the original ganglia dissociate. On the nonneuronal cell population no site I binding could be detected [2, 3]. In concordance

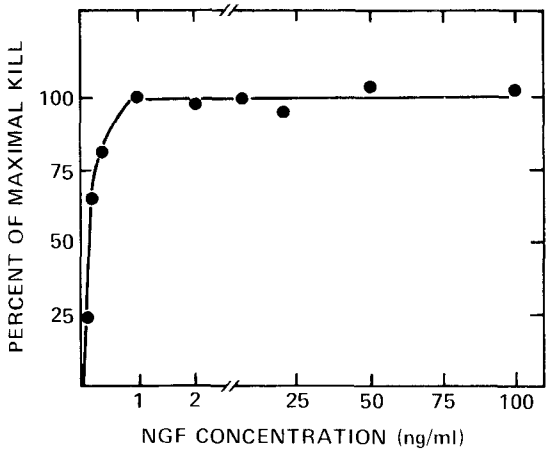

Fig. 2. NGF concentration dependence of cytotoxic kill. E8 sensory ganglia cells were preincubated with various NGF concentrations and then introduced into the cytotoxicity test. Concentrations given in the graph are final assay concentrations. Cytotoxic indices are expressed as percentage of the maximal obtainable kill. The cytotoxic indices were calculated in the following way:

$$ci = \frac{\%\text{ dead cells} - \%\text{ dead cells in controls}}{100\% - \text{dead cells in controls}} \times 100$$

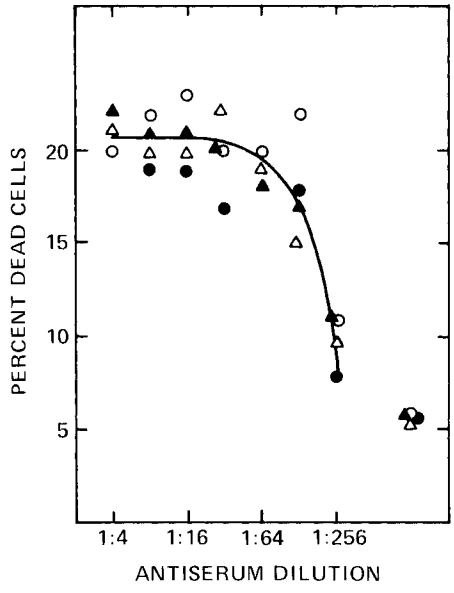

Fig. 3. Effect of washing on cytotoxicity. E8 sensory ganglia cells (6×10^6 cells/ml) were incubated with 4 ng/ml (●) and 70 ng/ml (▲) NGF for 30 min. They were then diluted 1:36, and incubated for another 10 min, following which they were spun down and resuspended in NGF-free medium to a concentration of 6×10^6 cells/ml. This cell suspension was used in the cytotoxicity test as described in Materials and Methods. Cytotoxic kill after preincubation with 4 ng/ml (○) and 70 ng/ml (△) without washing step. Symbols to the far right indicate the cytotoxicity in complement and serum controls.

with these results twice as many cells were killed when the neuronal population was tested in the cytotoxicity test in comparison to the original ganglia dissociate and no kill was observed on the nonneuronal cell population (see Fig. 5). When the neuronal cell population was cultured in the presence of NGF following the cytotoxicity assay, it was observed that the large, phase-bright, and NGF-responsive neurons were missing (Fig. 4d). For example, the average number of fiber-bearing neurons per visual field was reduced to 13 ± 4 (n = 8) following NGF-mediated cytotoxicity in contrast to 60 ± 15 (n = 8) in the controls (cytotoxic kill with anti-NGF antiserum and complement but without NGF). The type of fiber-bearing neurons observable in culture 24 h after NGF-mediated kill were very different from the large, NGF-responsive neuron type seen in Fig. 4b. They are much smaller and have shorter and more slender processes. While some of them are phase-bright, others appear dark. The dark type is recognizable on the photomicrograph of Fig. 4d, which was taken of a culture of neurons after cytotoxic kill with NGF, anti-NGF antiserum, and complement. Those neurons were also found to survive in cultures without NGF.

It has been shown previously that the sensory ganglia do not contain type I receptor-bearing cells during early embryologic development [2]. The same result has now been obtained using the cytotoxicity assay. Figure 6 shows the results of cytotoxicity tests on sensory ganglia cells from E4 and E8 chick embryos. No cytotoxic kill was observed when E4 sensory ganglia cells were incubated with NGF, anti-NGF antiserum, and complement.

Nonneural tissues lacking high-affinity receptors (I) for NGF should be insensitive to the action of anti-NGF antiserum and complement in the presence of NGF. As another control for the specificity of the NGF-dependent cytotoxicity assay cell suspensions of

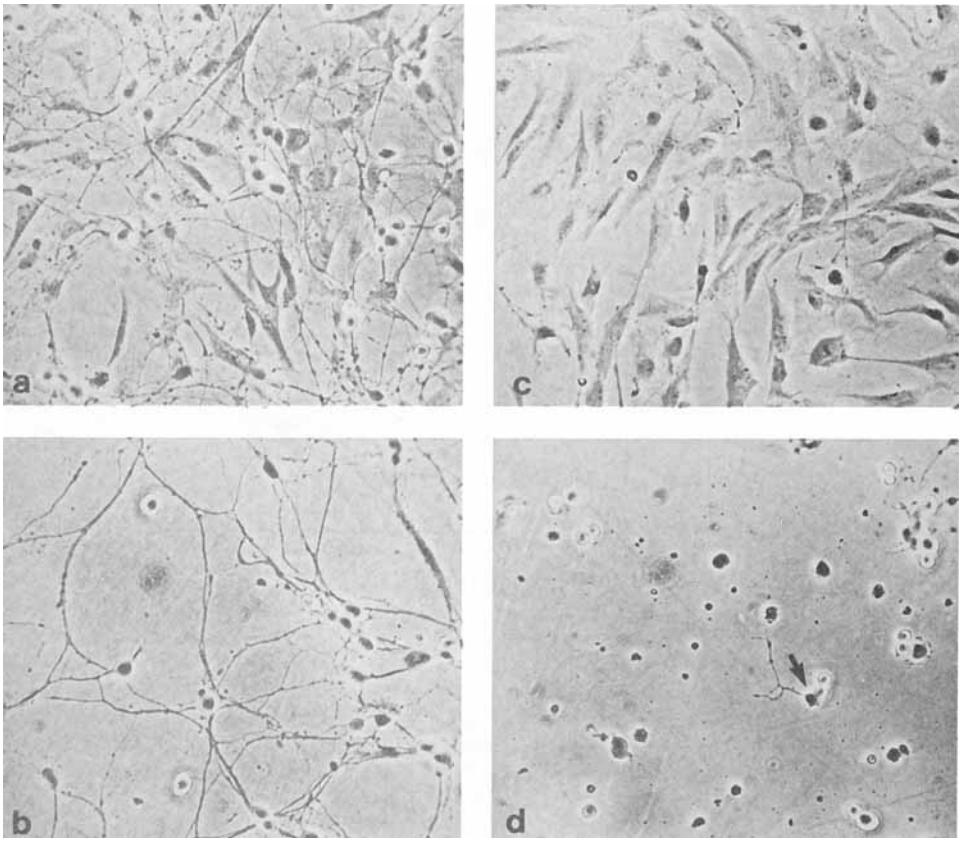

Fig. 4. Photomicrographs taken from 24-h cultures of a) ganglia dissociate, b) neuronal cells, c) non-neuronal cells, d) neuronal cells after NGF-dependent cytotoxicity (see Fig. 5). All cells were grown in the presence of 10 ng/ml NGF. The arrow in d) shows fiber-bearing neuron which survived the NGF-dependent cytotoxicity test.

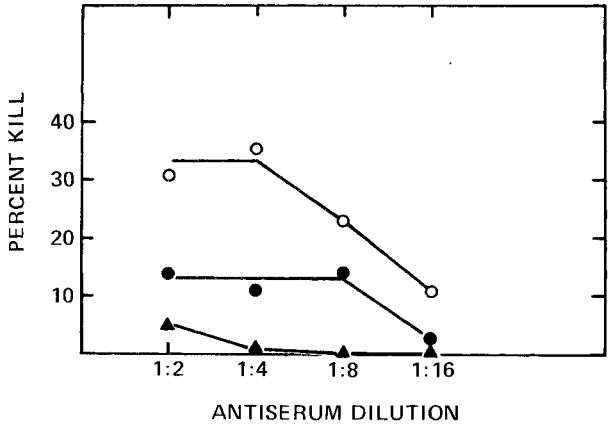

Fig. 5. Cytotoxic kill on the ganglia dissociate (•) and its neuronal (○) and nonneuronal cells (▲). The various cell suspensions were preincubated with 12 ng/ml before the test.

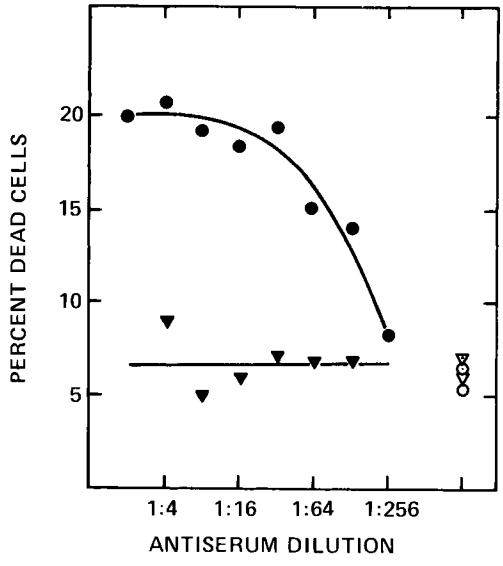

Fig. 6. Cytotoxic kill on sensory ganglia cells of two different developmental stages. The cell suspensions from E4 (▲) and E8 (●) were preincubated with NGF at a concentration of 12 ng/ml before the test. Serum controls (⊙ △), conplement controls (○ △).

heart and liver were tested. No cytotoxicity was observed on liver and heart cells prepared from E8 chick embryos (data not shown).

It is possible to monitor the high-affinity NGF-binding site (I) not only during embryonic development but also under culture conditions. Binding studies with ^{125}I-NGF have shown that type I and type II binding sites are differentially regulated in culture. Type I binding sites are down-regulated under certain culture conditions below the level of detectability, while the amount of type II sites increases dramatically (A. Sutter, unpublished data). Cytotoxicity assays on sensory ganglia neurons which had been cultured for 24 h show no NGF-dependent kill, which was to be expected in view of the drastic reduction or loss of the type I binding sites (data not shown).

The presence of NGF and its receptors in the central nervous system has been a point of debate for quite some time. We have analyzed the presence of high-affinity NGF receptors in the brain using the cytotoxicity assay. Figure 7 depicts the results of tests done on different brain parts of the E8 chick embryo. At this developmental stage a rather high percentage of forebrain cells can be killed. The kill is NGF-dependent (data not shown). The cytotoxicity on cells from the tectum is somewhat lower and no kill is observed on brainstem cells. We have further looked for the presence of type I receptor-bearing forebrain cells during development. The data are shown in Fig. 8. The cytotoxicity is the highest at E8 and E9 (the data for E9 are not shown in Fig. 8) of embryonic development. significantly lower at E10, and barely detectable at other stages like E5, E6, and E15.

DISCUSSION

Anti-hormone antibodies have been employed in hormone-receptor studies in several instances. For example, insulin receptors were localized with the aid of ferritin-labeled anti-insulin antibodies on adipocyte and liver membranes [8, 9], and the number of

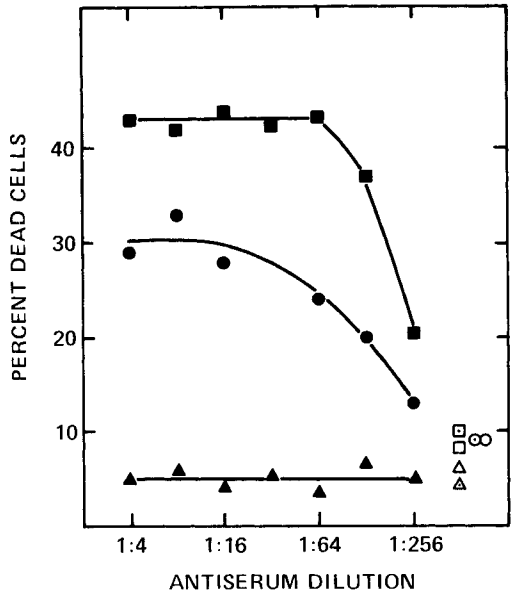

Fig. 7. Cytotoxic kill on various brain parts of the E8 chick. Cells from the forebrain (■), the tectum (●), and the brainstem (▲) were tested at an NGF concentration of 4 ng/ml. Serum controls (⊡ ⊙ ⧋), complement controls (□ ○ △).

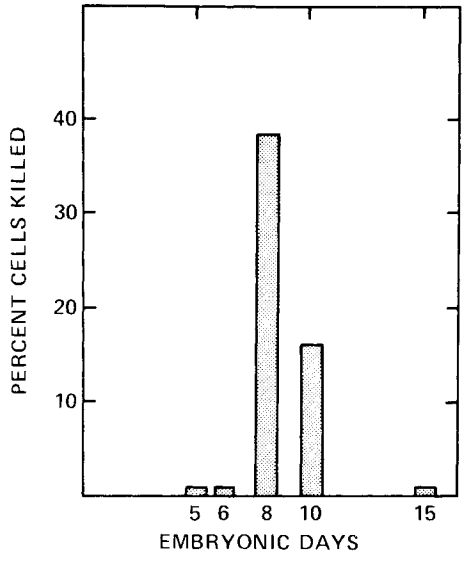

Fig. 8. Cytotoxic kill on forebrain cells at various developmental stages. Tests were done at an NGF concentration of 4 ng/ml.

epidermal growth factor (EGF) molecules bound and accessible on the surface of fibroblasts was quantified with ^{125}I-labeled anti-EGF antibodies [10]. As described here anti-NGF antiserum and complement were used to kill cells having NGF bound to their cell surface. The concentration dependence of NGF-mediated cytotoxicity suggests the use of this serological assay as a very sensitive procedure for the measurement of NGF concentrations in tissue homogenates, sera, conditioned media, etc. It is more sensitive than the NGF bioassay using whole ganglia [11], as sensitive as the radioimmunoassay [12], and faster though less sensitive than the single-cell bioassay [3]. We have presented evidence that only NGF binding to one of its two high-affinity receptors [2, 3], namely the one with the higher affinity, leads to cytotoxic kill of cells in the presence of anti-NGF antiserum and complement. The evidence includes the following findings: a) the concentration dependence of the kill (maximal cytotoxicity is obtained at an NGF concentration (1 ng/ml) at which hardly any type II sites are occupied) b) cytotoxicity assays including a washing step after preincubation of the cells with NGF (the washing step, while removing NGF bound to site II, does not affect the cytotoxic kill) c) the percentage of cells killed in the cytotoxicity assay is twice as high for the neuronal cell population as for the mixture of neurons and nonneuronal cells in the original ganglia dissociate. Correspondingly, binding assays have shown twice the number of type I binding sites on the neuronal cell population compared to the original dissociate [2, 3]. In addition to these experiments an NGF derivative in which 0.9 tryptophan residues per β-monomer were oxidized by N-bromosuccinimide, and which bound to the high-affinity site (I) with an affinity 20 times lower than that of native NGF, did not mediate cytotoxic kill [13].

Besides confirming the binding data which indicate that neurons and not the nonneuronal cells of the sensory ganglia carry NGF-binding sites of type I, the cytotoxicity assay has also supplied information about the number of type I receptor-bearing cells in the ganglia dissociate. At E8, about 20% of the cells in the ganglia dissociate and 40% of its neurons carry the high-affinity receptor site I. This statement is correct under the assumption that all cells having type I receptor can indeed be killed. Assuming that all neurons have about the same amount of site I on the cell surface and using the binding assay data [2, 3], one can calculate that each NGF-responsive neuron carries 15,000 high-affinity site I receptors for NGF. Occupancy of half or less of these receptors is sufficient for maximal kill.

Sensory ganglia contain two different classes of neurons: the "mediodorsal neurons" and the "ventrolateral neurons" [1]. Only the mediodorsal neurons seem to survive in cell culture in the presence of NGF. Using the cytotoxicity assay we could show that the NGF-responsive neurons carry the type I binding site for NGF. Clearly, the large, phase-bright, NGF-responsive neurons were missing in the cultures following the NGF-mediated cytotoxic kill. There were still a few fiber-bearing neurons present in the cultures after cytotoxicity. However, these were quite different in their morphology from the NGF-responsive neurons and they consistently survive in cultures of sensory ganglia cells grown in the absence of NGF. It is unclear so far which class of neurons these cells represent.

The cytotoxicity assay described here had been designed primarily with the objective of having a fast and easy assay to screen for the presence of the biologically relevant high-affinity receptors of type I in various tissues, cell lines, and especially parts of the central nervous system during development. At E4, an early stage of chick development, at which sensory ganglia cells do not respond to NGF in vitro and no high-affinity sites of type I are detectable in binding assays with ^{125}I-NGF [2], also no kill was observed in cytotoxicity

assays using NGF, anti-NGF antiserum, and complement although type II sites are present at this stage.

Presently the role of NGF in the central nervous system is still hypothetical [14]. The best evidence that NGF might also act in the central nervous system is derived from the observation that intracerebrally injected NGF promotes the regeneration of central norepinephrine-containing neurons [15]. We have not observed any effect of NGF on the survival or the morphological differentiation of brain cells in culture. Cells of the E8 forebrain of the chick were used in those experiments (A. Zimmermann, unpublished data). However, the cytotoxicity assay revealed the presence of type I NGF-binding sites on brain cells of the embryonic chick. When single-cell suspensions from brain regions of E8 chick embryos were analyzed, cytotoxic kill was obtained with forebrain and tectum cells but not with brainstem cells. A developmental study showed that E8 and E9 are embryonal stages with especially high numbers of type I receptor-bearing cells in the forebrain. The identification and isolation of type I receptor-bearing brain cells will be a part of an effort to determine the effect of NGF on those cells in vitro. Reports on NGF receptors in chick brain have been published previously [16]. They are based on ^{125}I-NGF-binding studies and apparently are at variance with our data based on cytotoxicity assays. They demonstrate specific binding of NGF to be about equally high on E13 forebrain, tectum, and brainstem. The cytotoxicity assay does not indicate the presence of any high-affinity receptors (I) for NGF on any of those brain regions at this developmental stage. The reasons for this discrepancy could be many. One is the possible differential susceptibility of different cell types to cytotoxic kill even though they may possess the same antigen (receptor) numbers. Another reason might lie in the different tissue preparations. Viable single-cell suspensions were used for the cytotoxicity assay, thereby allowing only the detection of NGF-binding sites on the cell surface of the cell body, while brain homogenates were used in the binding studies, which could include possible binding sites on neurites, synaptic endings, and nuclei. Further, the reports of specific NGF binding on brain homogenates as well as on homogenates of sensory and sympathetic ganglia do not differentiate between two independent binding sites. The NGF-binding data published for brain may therefore reflect mainly the binding of NGF to its type II binding sites, which constitute the majority of the specific NGF receptors on sensory ganglia cells rather than type I receptor binding which is responsible for the NGF-dependent cytotoxic kill.

An obvious advantage of the serological assay for NGF receptors is its sensitivity for the type I binding sites, which have been shown to be relevant sites for the NGF-induced neurite formation of sensory neurons. Binding of NGF to type II sites does not interfere with the assay. In principle, the assay developed for NGF and its receptor should also be applicable to other ligand-cell surface receptor systems provided that the receptor binding site of the ligand is not also an antigenic site. As demonstrated in this communication, kinetic parameters of ligand receptor interaction also may limit the use of this assay system.

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