Antibodies against mouse nerve growth factor interfere \textit{in vivo} with the development of avian sensory and sympathetic neurones

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Summary

The monoclonal antibody 27/21 directed against mouse nerve growth factor (NGF) interferes \textit{in vivo} with the survival of sensory dorsal root ganglion (DRG) neurones during the development of the quail embryo: the number of DRG neurones at embryonic day 11 (E11) was reduced by about 30\% in embryos treated with the antibody between E3 and E11. Neurone numbers in the nodose ganglion were not affected.

The effect of NGF antibodies on sympathetic neurones was assessed by determining the levels of the adrenergic marker enzyme tyrosine hydroxylase. Both total tyrosine hydroxylase activity and protein levels in sympathetic chains were reduced by about 30\% in embryos treated with 27/21 antibody but not in embryos treated with a control antibody.

The 27/21 antibody cross-reacts with chick NGF-like activity as shown \textit{in vitro} by the ability of the antibody to partially block the survival activity of chick-embryo-fibroblast-conditioned medium for E9 chick DRG neurones.

Key words: NGF, nerve growth factor, sensory neurone, sympathetic neurone, antibody, \textit{in vivo}, quail embryo, tyrosine hydroxylase, dorsal root ganglion.

Introduction

Nerve growth factor (NGF) is a protein required for the development and maintenance of specific populations of peripheral sensory and sympathetic neurones (Thoenen & Barde, 1980; Greene & Shooter, 1980; Thoenen \textit{et al.} 1987). It is synthesized by the corresponding peripheral targets of these neurones in very small quantities. The limited availability of NGF is thought to control the extent of neuronal survival and thus indirectly the density of innervation. Indeed, the density of innervation of several peripheral organs is correlated with the amount of NGF and NGF mRNA detectable in these tissues (Korsching & Thoenen, 1983; Shelton & Reichardt, 1984; Heumann \textit{et al.} 1984).

The biological importance of NGF has been demonstrated in mammals by the effect of antibodies directed against NGF, which leads to a complete destruction of sympathetic ganglia (Levi-Montalcini & Booker, 1960; Cohen, 1960) and to a reduction in the number of neurones in sensory dorsal root ganglia (Gorin & Johnson, 1979). The latter experiments are hampered by the fact that the antibodies reach the embryos only relatively late during development by transplacental transfer (Johnson \textit{et al.} 1980; Brambell, 1970). Thus it is not clear if at earlier embryonic stages other cells would also be affected by NGF. Indeed, it has recently been demonstrated that NGF receptors are present during very early development not only on classical target cells of NGF but also on spinal motor neurones and on muscle (Raivich \textit{et al.} 1985; Richardson \textit{et al.} 1986) suggesting a possible function of NGF in these systems. The ideal system to analyse these questions would be the chick embryo which is amenable to experimental manipulation from the earliest stages onwards.

Unfortunately, the antisera raised against mouse NGF and applied \textit{in vivo} to chick embryos had no effect on the development of sympathetic neurones (Levi-Montalcini, 1966; Hill & Hendry, 1981). \textit{In vitro}, however, chick NGF activity could be com-
Triton X-100 was added to a final concentration of 0-1% were homogenized and centrifuged as described earlier for the determination of NGF (Korsching & Thoenen, 1987). Glass beads (serving as the first site) as described in detail and the anti-TH antibody producing clone 2/40/15 (Rohrer et al.) have been determined by a two-site enzyme immunoassay al.

Levels of monoclonal mouse IgG antibodies in avian tissues have been determined by a two-site enzyme immunoassay (ELISA) using the principle of the assay developed previously (Korsching & Thoenen, 1987) for the determination of NGF. Affinity-purified polyclonal sheep antibodies (Bio-Science Products AG, FRG) directed against mouse IgG and unspecific sheep antibodies for the determination of unspecific binding were covalently coupled to glass beads (serving as the first site) as described in detail elsewhere (Korsching & Thoenen, 1987). Tissue samples were homogenized and centrifuged as described earlier for the determination of NGF (Korsching & Thoenen, 1987).

Depending on the expected IgG concentration, the supernatants were diluted with homogenization buffer and then Triton X-100 was added to a final concentration of 0-1% (w/v). The incubation and washing procedures were in principle those as described earlier (Korsching & Thoenen, 1987) with following modifications. A goat polyclonal anti-mouse-IgG-β-galactosidase conjugate (diluted 1:500; Medac, FRG) served as a second site. After 1–3h incubation at room temperature with 200 µM of the β-galactosidase substrate 4-methylumbelliferyl-β-D-galactoside, detection of the fluorescent reaction product permitted the determination of 0.2 ng of IgG per g and ml. A standard curve in the range of 0.2–20 ng IgG (monoclonal antibody 27/21) ml⁻¹ was determined for each assay. The data were corrected for recovery (82%), which was determined by the addition of a known amount of 27/21 antibody to the samples.

**Materials and methods**

**Antibody treatment**

Fertilized quail eggs were incubated at 37°C in a forced-draft incubator. On day 3, a window was cut into the shell and the shell membrane was removed. Antibodies were applied using two procedures.

1. 10 or 100 µg of purified monoclonal anti-mouse NGF antibody 27/21 were dissolved in phosphate-buffered saline, pH 7.4, and injected daily into the yolk sac of the embryos from embryonic day 3 to 10. The antibodies were purified by protein A-Sepharose chromatography from hybridoma supernatants.

2. 1 x 10⁶ cells of the anti-NGF antibody producing hybridoma clone 27/21 (Korsching & Thoenen, 1987) or of the anti-TH antibody producing clone 2/40/15 (Rohrer et al. 1986) were suspended in phosphate-buffered saline, pH 7.4, and applied at embryonic day 3 onto the chorioallantoic membrane. Both clones secrete antibodies belonging to the IgG subclass.

**Detection of monoclonal antibodies (mouse IgG) in avian tissues**

Levels of monoclonal mouse IgG antibodies in avian tissues have been determined by a two-site enzyme immunoassay (ELISA) using the principle of the assay developed previously (Korsching & Thoenen, 1983, 1987) for the determination of NGF. Affinity-purified polyclonal sheep antibodies (Bio-Science Products AG, FRG) directed against mouse IgG and unspecific sheep antibodies for the determination of unspecific binding were covalently coupled to glass beads (serving as the first site) as described in detail elsewhere (Korsching & Thoenen, 1987). Tissue samples were homogenized and centrifuged as described earlier for the determination of NGF (Korsching & Thoenen, 1987).

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**Histological procedures**

The 25th DRG (according to Palladini, 1961) and nodose ganglia (NG) were dissected from quail embryos at E11 (stage 28 according to Zacchei, 1961). The ganglia were fixed in 4% formaldehyde, embedded in paraffin, sectioned at 6 µm and stained with thionine. The neurones were identified by their large size and intensive staining and counted if they contained at least one nucleolus. Every other section was counted at a magnification of x250 as described by Hofer & Barde (1988).

**DRG cultures**

The cultures were established as described by Barde et al. (1980) with some minor modifications. Briefly, DRGs were dissected from E9 or E10 chick embryos, treated with 0-1% trypsin for 20 min at 37°C and dissociated by trituration. This cell suspension was enriched for neurones by the preplating procedure of McCarthy & Partlow (1976) in which non-neuronal cells are removed by allowing them to adhere to the surface of a tissue culture dish. The neurone-enriched cell suspension was plated in 24-multiwell tissue culture dishes (Costar) that had been coated with polyornithine (Sigma) and Schwannoma-conditioned medium (Edgar et al. 1988) at a density of 6000 cells per well.

The response of dissociated neurones to NGF or CEF-CM was assessed by counting the number of process-bearing neurones (bright-phase neurones with neurites at least two cell diameters in length) 24–48h after plating.

NGF was purified from mouse salivary glands according to Bocchini & Angeletti (1969) as modified by Suda et al. (1978) using a 55% saturation ammonium sulphate precipitation.

The chicken embryo fibroblast medium (CEF-CM) is a conditioned medium of spontaneously transformed chicken embryo fibroblasts, cell line CEC-32, clone LSCC-H32 (Kaaden et al. 1982).

Monoclonal anti-mouse NGF antibody 27/21 used for the in vitro experiments was purified by protein A-Sepharose (Pharmacia) chromatography from hybridoma supernatants.

Neurotrophic factors and antibodies were added simultaneously to the cell suspension before incubation.

**Determination of tyrosine hydroxylase (TH) in sympathetic chain ganglia**

Sympathetic chains were dissected from E11 embryos and
kept frozen at −70°C until further use. TH enzyme activity was determined as described previously (Acheson et al. 1984). Protein content was determined by the Bradford procedure (Bradford. 1976) with ovalbumin as standard.

Results

Antibody concentrations reached within the chick embryo depend on the mode of application

We determined the amount of antibody present in the embryos after different injection protocols by a specific immunological detection method (ELISA). Quail embryos were used because the volume of the quail eggs is considerably smaller than that of chick eggs and data were available on the numbers of sensory DRG and NG neurones during development (Hofer & Barde, 1988). Monoclonal anti-NGF antibodies (27/21) were injected daily into the yolk sac of quail embryos between E3 and E11. Daily injections of 10 μg of antibody (27/21) resulted in antibody concentrations of about 0.017 ± 0.007 μg g⁻¹ wet weight (n = 8) in brain or heart tissues (no difference). After injections of 100 μg day⁻¹ the concentrations were increased to 0.7 μg g⁻¹ wet weight (mean of two determinations). It should however be noted that only a very small proportion of the injected antibody could be detected in the embryos. Antibody injections into the yolk sac seem to be a very inefficient method of antibody application.

As an alternative way of antibody application, the antibody-producing hybridoma cells were applied onto the chorioallantoic membrane of E3 quail embryos. This resulted consistently in higher levels of antibodies both in brain and heart (3 ± 1.4 μg g⁻¹ wet weight in the case of clone 27/21) and was therefore used in the experiments described below.

Effect of anti-NGF antibody 27/21 on the number of sensory neurones in quail DRG and in the nodose ganglion (NG)

The number of neurones in DRG and NG during the development of the quail embryo has recently been determined by Hofer & Barde (1988). After periods of neuronal death, neuronal cell numbers reach constant levels at E11 in both DRG and NG. Thus the effect of anti-NGF antibody 27/21 on the survival of sensory neurones was assessed by determining the number of ganglion neurones at E11 both in DRG and NG. In the embryos treated with anti-NGF antibodies, the number of neurones in DRG was decreased by 26% (Table 1). In the same embryos, the number of neurones in NG was not affected by the treatment. In order to exclude an unspecific effect of antibodies on the survival of DRG neurones, embryos were also treated with monoclonal antibodies against tyrosine hydroxylase (clone 2/40/15; Rohrer et al. 1986), an intracellular protein. Although the antibody concentration in the embryo reached even higher values (29 ± 3 μg g⁻¹ wet weight), the anti-TH antibodies did not affect the number of sensory DRG neurones (Table 1).

Effect of anti-NGF antibody 27/21 on sympathetic ganglion neurones

TH catalyses the rate-limiting step in the synthesis of catecholamines and is often used as marker enzyme for adrenergic sympathetic cells. In the rat, the effect of anti-NGF antibodies on sympathetic ganglia has been analysed previously by measurement of TH activity (Goedert et al. 1978; Gorin & Johnson, 1979; Johnson et al. 1980) and has been shown to correlate during development with effects on the cell number (Johnson et al. 1980).

The TH activity of lumbosacral paravertebral sympathetic chains from E11 quail embryos treated with anti-NGF antibody 27/21 was reduced by 32% (Fig. 1). Concomitantly with the loss of TH activity the protein content of the chains was also reduced (29%). The effect was specific for anti-NGF antibodies since treatment of embryos with anti-TH antibodies reduced neither TH levels nor protein levels. In fact TH and protein levels were slightly higher than in uninjected control embryos (116 ± 11% and 105 ± 10%, respectively).

Table 1. Effect of anti-NGF antibody 27/21 on the number of sensory neurones in DRG and NG

<table>
<thead>
<tr>
<th>Ganglion</th>
<th>Treatment with antibody</th>
<th>Neurone number</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRG</td>
<td>no treatment</td>
<td>4336 ± 184 (n = 5)</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>anti-NGF (27/21)</td>
<td>3205 ± 354 (n = 8)</td>
<td>73.9%*</td>
</tr>
<tr>
<td></td>
<td>anti-TH (40/15)</td>
<td>4599 ± 629 (n = 4)</td>
<td>106%</td>
</tr>
<tr>
<td>NG</td>
<td>no treatment</td>
<td>3557 ± 447 (n = 4)</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>anti-NGF (27/21)</td>
<td>3794 ± 368 (n = 4)</td>
<td>106%</td>
</tr>
</tbody>
</table>

* Significantly different from untreated controls (P < 0.0005) and from anti-TH treated embryos (P < 0.001).

Anti-NGF antibody 27/21 interferes with NGF-like neurotrophic activity in chick-embryo-fibroblast-conditioned medium (CEF-CM)

In order to demonstrate further the cross-reactivity.
Fig. 1. Effect of anti-NGF antibodies on TH enzyme activity (A) and protein content (B) of sympathetic ganglia. Embryos were injected with antibody-producing hybridoma cells at E3. Sympathetic ganglia were dissected at E11 and assayed for TH activity and protein content. Uninjected control embryos; embryos injected with anti-NGF-producing hybridoma cells. The values given are the means ± S.E.M of three independent experiments each containing four to eight embryos. * Significantly different from controls (P<0.05).

between avian NGF and the anti-NGF antibody 27/21, chick-embryo-fibroblast-conditioned medium (CEF-CM) was used as a source of avian NGF-like neurotrophic activity (Young et al. 1975). Preliminary experiments have shown that CEF-CM from CEC-32 cells (clone LSCC-H32, Kaaden et al. 1982) contained considerable amounts of survival-promoting activity for E9 chick DRG neurones. Here we demonstrate that about 70% of E9 DRG neurones could be maintained in vitro in the presence of CEF-CM whereas only about 55% of the cells survive with NGF (Fig. 2).

The addition of anti-NGF antibody 27/21 resulted in a reduction of the number of surviving neurones. However, the survival effect of CEF-CM could be only partially blocked by anti-NGF. About 30% of the neurones plated survived even in the presence of a large excess of anti-NGF antibody. These results indicate that CEF-CM contains at least two factors: a NGF-like survival factor which is blocked by anti-NGF 27/21 and additional survival factor(s) which is not blocked.

Discussion

The present results demonstrate that NGF is required in vivo for the development of peripheral avian sensory and sympathetic neurones. Administration of a monoclonal antibody directed against mouse NGF between embryonic days 3 and 11 specifically pro-

Fig. 2. (A) Dose–response of E9 DRG neurones to mouse NGF. Neuronal survival was determined after 2 days in culture. Values given are the means ± S.E.M. of three to five independent experiments. (B) Dose–response of E9 DRG neurones to CEF-CM. Neuronal survival was determined after 2 days in culture. Values given are the means ± S.E.M. of three to five independent experiments. (C) Anti-mouse NGF antibody inhibits neuronal survival effects of mouse NGF and CEF-CM. Antibodies were added simultaneously with the survival factors at the beginning of the culture. NGF was present at 1 ng ml⁻¹, CEF-CM was used at a dilution of 1:2. Survival was assayed after 2 days in culture. Values given are the means ± S.E.M. of three to five independent experiments.
produces a reduction in the number of sensory DRG neurones but does not affect neurone numbers in the nodose ganglion.

The naturally occurring neuronal cell death in sensory ganglia of the peripheral nervous system is thought to be due to the limited availability of neurotrophic factors present in their peripheral and central target fields. It has been demonstrated indeed that the death of quail sensory DRG neurones can be prevented in vivo by the administration of NGF or of brain-derived neurotrophic factor (BDNF) (Hofer & Barde, 1988). There is considerable evidence that sensory neurones from DRG and NG differ in their response to NGF. DRG neurones express receptors for NGF (Sutter et al. 1979; Rohrer & Barde, 1982) and respond to NGF both in vivo (Hamburger et al. 1981) and in vitro (Levi-Montalcini & Angeletti, 1963; Barde et al. 1980). In contrast, avian NG neurones do not express NGF receptors (Lindsay & Rohrer, 1985) and their survival is influenced by NGF neither in vivo (Lindsay & Rohrer, 1985) nor in vitro (Dimberg et al. 1987; Hofer & Barde, 1988).

Our demonstration that antibodies against mouse NGF interfere with the survival of sensory DRG neurones but not with the survival of NG neurones supports those previous results. Since NG neurones are not influenced, we conclude that the effect of the anti-NGF antibody is not due to unspecific damage of cells but rather due to the elimination of endogenous NGF. This conclusion is further supported by the lack of effect of control antibodies. In mammals, the sympathetic ganglion neurones are also affected by the administration of anti-NGF antibodies either pre- or postnatally (Cohen, 1960; Levi-Montalcini & Booker, 1960; Gorin & Johnson, 1979). Cell numbers and the levels of TH, the marker enzyme of adrenergic neurones, are dramatically reduced (Gorin & Johnson, 1979; Johnson et al. 1980). We used the determination of TH to monitor the effect of anti-NGF antibodies on chick sympathetic neurones. In mammals, it has been observed that there is a good correlation between the effect of anti-NGF antibodies on TH levels and neurone numbers (Johnson et al. 1980). In the present study, we also observed a reduction in the levels of TH and protein of lumbosacral sympathetic chain ganglia in embryos containing anti-NGF antibody.

The effect of the monoclonal anti-NGF antibody on both sympathetic and sensory ganglia was relatively small and amounted to about 30% in both cases. In contrast, in mammals both sensory and sympathetic neurones can be eliminated virtually completely (Levi-Montalcini & Booker, 1960; Gorin & Johnson, 1979; Johnson et al. 1980). The small effects of anti-mouse NGF antibody in quail embryos can be explained (as in the previous experiments where no effect at all had been observed (Levi-Montalcini, 1966; Hill & Hendry, 1981)) by reduced cross-reactivity of the anti-mouse NGF antibody with chick NGF. From our in vitro experiments, using CEF-CM as a source of avian NGF, we can conclude that a NGF-like activity present in CEF-CM can be completely blocked by an excess of the antibody. Similar results have been obtained using chick-heart-cell-conditioned medium (Norrgren & Ebendal, 1986). However, the extent of cross-reactivity remains unclear since the amount of chick NGF present in the conditioned media is not known. It is not possible to determine the amount of NGF by assaying the survival effect since conditioned media contain a mixture of different factors affecting neuronal survival (Barde et al. 1978; Collins, 1978; Helfand et al. 1978; Varon & Adler, 1981) which may interact to potentiate the survival effect of NGF (Edgar & Thoenen, 1982; Davies et al. 1986). Ebendal and co-workers have recently succeeded in isolating biologically active NGF from chick embryos (Ebendal et al. 1984; Belew & Ebendal, 1986) and evidence has been presented that polyclonal anti-mouse NGF antibodies cross-react only partially with chick NGF (Belew & Ebendal, 1986). But it remains unclear to what extent the antigenicity of chick NGF was altered by the purification procedure. There is evidence that the immunological properties of bovine and mouse NGFs differ substantially (Harper et al. 1983) although the similarity between bovine and mouse NGFs, as deduced from DNA sequence analysis, is very high and amounts to 84% (Meier et al. 1986). Thus, a reduced cross-reactivity at least of the polyclonal antisera would also be expected for chick and mouse NGFs, which are even less similar than bovine and mouse NGFs (19 as compared to 16 amino acid substitutions) (Meier et al. 1986; Ebendal et al. 1986). Although the cross-reactivity of the anti-mouse NGF antibody 27/21 with chick NGF is not known at present, it is important to point out that, in excess of the antibody, a plateau was reached, indicating a complete inhibition of chick NGF. Since chick NGF (in CEF-CM) and anti-NGF antibodies were added simultaneously to the cells it has to be concluded that the antibody interferes directly with the action of NGF.

The question remains, however, whether the concentrations of the antibody in the embryo were high enough to block the action of endogenous NGF completely. In the present experiments, the antibody concentration was about 3μg g⁻¹ wet weight at the end of the incubation time. Under the assumption that NGF is present in chick tissues at similar concentrations as in rat and mouse tissues (1–4ng g⁻¹ wet weight in heart or iris) (Korsching & Thoenen, 1983; Heumann et al. 1984), the antibody concentrations would be 5- to 20-fold in excess of the amount...
necessary to block the effect of NGF (assuming a cross-reactivity between chick and mouse NGF of 1:3). In rats, strong effects of anti-mouse NGF antibodies were observed at antibody concentrations which are 10- to 50-fold in excess (assuming 1 ng NGF g⁻¹ tissue and assuming that the antibody concentration in serum and tissue are identical) (Gorin & Johnson, 1979, 1980). It is conceivable, however, that the antibody concentration during the first days of incubation was too low and/or that the local concentrations of NGF in target tissues are too high to ensure a complete block of NGF effects. The latter possibility seems not to be very likely since the levels of NGF mRNA in chick tissues are very similar to those of rat tissues (Ebendal et al. 1986; Rohrer et al. 1988). An alternative possibility would be that in the chick, in contrast to mammals, the survival of some sensory and sympathetic neurones is controlled by survival factors different from NGF. In this case, only the combined elimination of NGF and of the other factors would result in the death of all sensory and sympathetic neurones.

In order to analyse those questions in more detail, the amounts of chick NGF have to be determined and an antibody has to be used with a known cross-reactivity to chick NGF. From the data presented here, we can conclude that NGF is of physiological importance at least for some sensory and sympathetic neurones.

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References


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