Immunology of nerve growth factor (NGF). The effect of NGF-antiserum on sensory ganglia in vitro

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The properties of the 'Nerve Growth Factor' (NGF) have been described extensively (Levi-Montalcini & Booker, 1960; Levi-Montalcini, 1965) and reviewed recently (Levi-Montalcini, 1966). This factor is a protein of molecular weight about 130000 in its aggregated form (Varon, Nomura & Shooter, 1967, 1968) but may be active in lower molecular weight forms (Cohen, 1959, 1960; Banks et al. 1968). It is widely distributed in the adult organism (Bueker, Schenkein & Bane, 1960) and exerts a controlling influence on the differentiation of sensory and sympathetic ganglia in developing chick embryos. In newborn mammals its administration influences sympathetic ganglion growth only. A possible role in the adult nervous system is suggested by Scott, Gutmann & Horsky (1966), who showed that injected NGF will increase protein synthesis in regenerating sensory neurons in vivo.

Active proteins in complex biological systems may be removed in a highly selective fashion by specific antibodies which thus provide a valuable means of studying their action. Cohen (1960) prepared an antiserum against purified NGF, and found that it inhibited the action of NGF on chick ganglia in vitro, and caused marked reduction in postnatal ganglionic development when injected into newborn mice. Levi-Montalcini & Angeletti (1960) further extended these studies, finding that when administered to newborn mammals, the antiserum almost totally depopulated the sympathetic ganglia. When injected into young adults the antiserum reduced their ganglion cell population by 60 %. Later workers (Vogt, 1964; Zaimis, Berk & Callingham, 1965) found that the serum acted selectively: whilst the paravertebral ganglia were largely depopulated, the prevertebral ones were either unaffected (mesenteric) or only partially destroyed (coeliac).

Only limited information is available concerning the action of NGF antiserum on chick ganglia in vitro. This is partly due to the behaviour of ganglia when grown on glass surfaces, where the mass of cells remains coherent, and a

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halo of neurites emerges around it. Antiserum action has been demonstrated as a suppression of the halo appearance, and titre measured by diluting until a concentration is reached in which the neuritic halo again grows out.

Little has been learned about the action of the antiserum in vitro on individual cells: Sabatini, Pellegrino de Iraldi & de Robertis (1965) studied the changes in fine structure of ganglion cells treated with antiserum and observed degenerative changes in the first few hours. They suggested that the action of the antiserum, which appeared to be complement dependent, was due to antigen-antibody reaction within the cytoplasm.

The destruction, by NGF antiserum, of ganglion cells which would have been stimulated to more rapid and extensive differentiation by NGF, may seem surprising. It can hardly be regarded as a simple interaction between antigen and antibody. Levi-Montalcini & Angeletti (1963) showed that trypsin-dissociated chick ganglion cells will not survive when incubated in defined synthetic media such as Eagle's solution. If NGF is added to the medium they will survive and grow indefinitely. The authors concluded that NGF was a vital growth requirement of the cells. If this is so, it is tempting to speculate that antibody might destroy the neuroblasts simply by removing this vital growth factor.

The action of NGF antiserum on isolated ganglion cells has been described briefly in an earlier paper (Hoffman & McDougall, 1968). The experiments described below were designed to elaborate and elucidate further the action of this antiserum on neuroblasts in vitro. The experimental procedures used here are those described in our previous papers (Hoffman, Naughton, McDougall & Hamilton, 1967; Hoffman & McDougall, 1968), which were developed in order to permit the study of individual neuroblasts migrating freely over acrylamide gel surfaces.

MATERIALS AND METHODS

Sensory ganglia were obtained from 10- to 11-day incubated chick embryos, then transferred on to discs of acrylamide gel, placed together with medium within teflon rings waxed to microscope slides and sealed on top with coverslips, as described in our earlier papers.

The media used were either fowl plasma, prepared with EDTA and clotted with minimal thrombin containing sufficient Ca^{2+} to restore the original ionic concentration, fowl serum, or defined liquid media such as Eagle's solution or Medium 199. The volume used was usually ca. 0.3 ml. The acrylamide gel discs were cut with a cork borer from sheets of gel, prepared by polymerizing a 2 mm thick layer of acrylamide (7\% \%) in a Petri dish under a layer of water. Gel discs were soaked for \ \frac{1}{2} \ h in Hanks's solution containing 0.01 \% terramycin (oxytetracycline) before use.

Primary and secondary antisera were prepared in rabbits, using a purified particulate component obtained from male mouse submaxillary gland homogenate. This NGF preparation revealed five separate bands electrophoretically,
three of which proved to have NGF activity when tested on electrophoretic gels as described by Hoffman & McDougall (1968): it produced detectable growth promotion in ganglion cultures in a concentration of 1 μg/ml.

Samples of these sera were added in graded dilutions to the medium in which the ganglia were explanted and the culture cells were then sealed and incubated for 24-48 h in an atmosphere of 95% oxygen, 5% carbon dioxide. The culture cells were then removed, teflon rings and coverslip discarded, another coverslip applied directly to the culture, and the whole examined with phase contrast optics.

A proportion of the control and experimental cultures were fixed in Formol-Muller solution, for paraffin embedding, then sectioned and stained with Giemsa or Pyronin-Methyl green. Other cultures were fixed with glutaraldehyde, followed by osmium tetroxide, embedded in Epon, and sectioned. The sections were stained with uranyl acetate, followed by lead citrate, for electron microscopy.

Ganglia were incubated, in one series of experiments, in culture medium containing 'Difco' trypsin, in concentrations 1–10⁻² mg/ml. Batch no. of the trypsin was 486425.

RESULTS

(1) Growth of ganglia in normal media

(a) Synthetic medium

Fig. 2 illustrates the extent and character of the cellular migration from ganglionic explants on gel, when incubated in Eagle's solution. Under control conditions there was considerable movement of cells from the ganglionic mass out on to the gel surface. A wide variety of cell types was seen, ranging from large definitive neuroblasts (arrows) averaging 20–25 μ in diameter such as are seen in Figs. 1, 2 and 4, some extruding neurites as in Fig. 4 (arrow), through partially differentiated neuroblasts of about 8–10 μ in diameter to much smaller 'embryonic' or undifferentiated cells around 4–5 μ in diameter such as seen in Fig. 9 (arrows). Commonly the differentiated and differentiating cells preponderated in the cell population. The nature of the small 'embryonic' cells will be discussed later in this paper.

Growth in Medium 199 was almost identical in every respect to growth in Eagle's solution.

(b) Plasma clot

The character of outgrowth of cells from ganglionic explants in a plasma clot is illustrated in Fig. 1. Here there was rather less migration, but the cell population was almost exclusively composed of large neuroblasts (arrows). The tiny 'embryonic' cells seen in defined medium cultures were absent, and smaller neuroblasts were rarer. Intermittently a neuroblast sprouting a neurite was encountered.
Growth of ganglia in medium containing antiserum

(a) Eagle's solution or Medium 199

The presence of NGF antiserum in titres of $10^{-1}$–$10^{-3}$ in synthetic culture medium resulted in a total absence of mature neuroblasts from the cell population moving out from the ganglionic explants. As is shown in Figs. 10 and 12, a mixture of smaller cells emerged, in which the diminutive 'embryonic' or undifferentiated cells predominated (arrows). These cells, averaging about 4 μ in diameter, have a comparatively large, bright, refractile nucleus, often with conspicuous nucleolus, surrounded by a thin rim of dark hyaline cytoplasm. Partially differentiated neuroblasts (n) were present in smaller numbers (see Fig. 10) and in these could be seen granular cytoplasmic components. The 'embryonic' cell type is particularly well illustrated in Fig. 12 in which almost every cell seen belongs to this class. As the concentration of antiserum fell ($10^{-4}$–$10^{-6}$) larger neuroblasts appeared amongst the smaller cells, illustrated in Fig. 8. At about $10^{-6}$ concentration of antiserum the population approached that seen in control cultures.

The diminutive 'embryonic' cells seen in such considerable numbers spreading over the gel surface when ganglia were explanted in Eagle's solution containing $10^{-1}$–$10^{-3}$ antiserum had a very characteristic appearance; they differed markedly from the more mature neuroblasts, and from satellite cells, especially in their nucleocytoplasmic ratio: this ratio is very high in the case of the undifferen-
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tiated cells. These small undifferentiated cells, when seen in stained sections of cultures in the light microscope have a narrow rim of quite basophilic cytoplasm, which in the electron microscope is seen to contain numerous ribosomes.

They nearly always occurred together with cells of various sizes and stages in the maturation of neuroblasts. Occasionally some of these tiny cells extruded processes resembling the neurites of neuroblasts.

Small numbers of these immature cells could always be found in cultures grown in synthetic media: they resembled the embryonic cells seen migrating from cultures of chick neural tubes incubated for 48 h, illustrated in Fig. 18 (arrows). Similar cells can be seen in sections of sensory ganglia of very early chick embryos: in Fig. 16 a section of 4-day chick sensory ganglion is shown, and many such cells of average diameter 4 μ are seen. In Fig. 14, a section of sensory ganglion from 10-day chick, few such small cells appear—the cell population is composed almost entirely of mature neuroblasts. Fig. 13 is a phase contrast photograph of a fresh sensory ganglion from a 10-day chick, and in this figure a few small cells of the embryonic type described above may be detected (arrows).

Thus the cell population seen on the gel or in the ganglia after incubation in fairly concentrated NGF antiserum in synthetic medium was very different from that present in the ganglia before incubation.

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Fig. 8. Population of cells which have migrated on to the gel surface from a ganglion of 11-day chick, incubated in Eagle's medium containing 10^{-6} concentration of NGF boosted antiserum. This is a mixed population of cells, mostly dense spherical neuroblasts (n), but there are a few tiny 'embryonic' cells indicated by arrows. Phase contrast. × 470.

Fig. 9. Spread of mixed population of cells from a ganglion of 10-day chick incubated 48 h in Eagle's medium without antiserum. As in the previous figure there are large neuroblasts (n) accompanied by numerous small cells (arrows). Phase contrast. × 470.

Fig. 10. Cell population from a culture in Eagle's medium containing a higher concentration of boosted antiserum than in Fig. 8. The concentration of antiserum here (10^{-3}) has resulted in reduction of the numbers of mature neuroblasts, and the predominant element now is the tiny 'embryonic' element indicated by arrows. Phase contrast. × 470.

Fig. 11. Cell population seen growing out from a ganglion incubated in primary antiserum diluted in plasma to 10^{-6}. Here only mature cells are seen, and the proportion possessing neurites (arrows) is greatly in excess of control cultures. This represents a fairly considerable concentration of NGF in the medium. Lysis is absent, indicating that dilution has eliminated lytic capacity. Phase contrast. × 470.

Fig. 12. The cell population shown here, around the edge of the ganglion, has migrated in Eagle's medium containing 10^{-4} concentration of boosted antiserum. Here every cell visible is in the 'embryonic' category (arrows). Phase contrast. × 470.

Fig. 13. This figure shows a phase contrast picture of a flattened live ganglion just removed from a 10-day chick. Although the ganglion contains a few 'embryonic' cells (arrows) the majority are mature large neuroblasts (n). × 470.
(b) Plasma

With the addition of antiserum to clotted EDTA plasma medium, rather less migration of neuroblasts was observed. Usually a ring of cells was formed, several layers thick, around the ganglion, and within 24 h most or all of these cells were lysed, in a characteristic fashion, resembling haemolysis in erythrocytes. The cells appeared in phase contrast as bright, hyaline 'ghosts' as shown in Figs. 3 and 5. In Fig. 3, as well as numerous 'ghosts' (g) several more or less intact neuroblasts (n) may be detected, together with cellular debris (d). Occasionally the lysis was incomplete; migration and neurite formation sometimes preceded lysis and such appearances as in Figs. 6 and 7 were observed. In both these figures the cell bodies (n, nl) are partially lysed, and the neurite (ne) is represented by a chain of granules. In some atypical instances 'ghosting' was not observed, the neuroblasts appeared to explode. Usually only relatively mature neuroblasts were lysed, cells in early stages of differentiation, and undifferentiated embryonic cells were unaffected.

Typically, when our secondary antisera were used in experiments, lysis was observed in medium containing $10^{-1}$–$10^{-5}$ or $10^{-6}$ concentration of antiserum. (Certain sera fail to lyse at $10^{-1}$ concentration, but lyse from $10^{-2}$ downwards. This is regarded as a form of 'prozone'.) Usually, at the terminal dilution, lysis was infrequent, or incomplete in many cells. Thus, at terminal titre, normal migration and differentiation of neuroblasts began to be observed. Only at this terminal dilution of antiserum did neuroblasts sprout neuritic processes.

Fig. 14. Cell population in a ganglion similar to that seen live in the previous figure. As in the live ganglion it will be noted the cells are mostly maturing neuroblasts. From a 10-day chick fixed in Bouin's solution, stained Giemsa's stain, x 470.

Fig. 15. ‘Woolly’ degeneration of neuroblasts (n) incubated in plasma containing $10^{-4}$ concentration of trypsin. This degeneration progresses slowly until the cell disintegrates. Phase contrast. x 470.

Fig. 16. Cell population in a ganglion from a 4-day chick embryo. Here all the cells are of the embryonic neural type, closely resembling the small cells seen in earlier figures. Bouin's solution fixation, Giemsa's stain. x 470.

Fig. 17. More severe ‘woolly’ degeneration of neuroblasts (n) incubated in plasma containing $10^{-3}$ concentration of trypsin. Phase contrast. x 470.

Fig. 18. Typical small embryonic neural cells which have streamed out of an explanted neural tube of 2-day chick, in plasma on an acrylamide gel surface. Arrows point to cells which resemble closely the small ‘embryonic’ cells seen in cultures of ganglia in Eagle's medium and antiserum. Although these cells seen here are a little larger, their cytological character is quite similar to those seen in earlier figures illustrating ganglionic cell populations. Phase contrast. x 470.

Fig. 19. This figure shows the edge of a ganglion, lined with lysed neuroblastic ‘ghosts’ (g), after incubation for 48 h in plasma containing heparin at a concentration of 250 i.u./ml. This lysis appears essentially similar to that induced by antibody in plasma. Phase contrast. x 470.
(3) **Ganglionic growth in synthetic media containing serum**

Whilst the occurrence of lysis of neuroblasts in the natural media might be expected from the *in vivo* results mentioned in the introduction, the failure of lysis, and the apparent arrest and reversal of differentiation produced by antiserum in synthetic medium were rather unexpected. In order to explore this phenomenon further, ganglia explanted in pure serum, 10% serum in Eagle's solution, 3% serum in Eagle’s solution and pure Eagle's solution were compared. In addition, antiserum was diluted serially $10^{-3}$–$10^{-6}$ in each of these media.

Fig. 20. A map of the stained protein bands in an analytical acrylamide gel of fowl serum. The $R_F$ (Reduction factor) values are indicated on the left. When ganglia were incubated in Eagle's solution containing $10^{-3}$ of NGF-antiserum, immediately above this gel, lysis of neuroblasts occurred appreciably at $L$, and very slightly at $I$.

Neuroblasts grew differently in the various culture media. Growth and migration proceeded most actively in Eagle's solution, least actively in serum, while the addition of serum to Eagle's solution decreased the growth activity, in proportion to the concentration of serum added. Growth in serum differed little in level from growth in plasma. When antiserum was added to the various media, the effects on cultures were graded characteristically. Antiserum in pure Eagle’s solution produced the same non-lytic dedifferentiation pattern as described earlier: antiserum in pure serum produced lysis similar to, and with the same graded effect with dilution, as the lysis produced by plasma in the earlier experiments. Dilutions of antiserum in Eagle’s solution containing 10% of
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serum produced results quantitatively indistinguishable from those obtained with antiserum in pure serum, while similar dilutions of antiserum in Eagle's solution containing only 3% of serum produced less complete lysis, waning earlier in the dilution series than was observed with pure or 10% serum, where lysis disappeared at a dilution of $10^{-3}$ antiserum.

(4) Localization of the lytic cofactor on electrophoretic gel

From the results presented in the previous section, it might be concluded that serum contains a cofactor required by antibody in its lytic role. The requirement of serum as a cofactor for lysis might suggest a complement-fixation cytolytic antibody mechanism; however, the addition of 3% guinea-pig serum to Eagle's solution containing NGF antiserum did not induce lysis. Moreover, mammalian antibody generally will not fix avian complement—thus complement-fixation would not occur in the system being observed here. In order to study further the cofactor apparently involved in the lysis of neuroblasts, fowl serum was electrophoretically distributed in an acrylamide gel, ganglia were arranged along a thin longitudinal slice of this gel, using a technique described earlier (Hoffman & McDougall, 1968; Hoffman et al. 1967) and the gel was covered with Eagle's solution containing $10^{-2}$ concentration of antiserum, which was then sealed and incubated. Most of the ganglia settled and spread on the gel, and growth was of the character already described for antiserum in Eagle's solution. In one region, illustrated in Fig. 20, at $R_F$ 0.47 considerable, typical lysis occurred, while a trace of lysis could be detected at $R_F$ 0.04 and 0.68. On the basis of the previous experiments, lysis would occur where the antiserum was supplemented by a serum-borne cofactor; thus, more than one component of serum appears to act as cofactor.

(5) Action of trypsin on neuroblasts

All of the evidence relating to lysis of neuroblasts presented here favours the suggestion of Sabatini et al. (1965) that NGF antibody (supplemented by a serum cofactor) directly lyses neuroblasts.

Cell damage produced by antibody to NGF may result because the antibody removes from the cell what Levi-Montalcini & Angeletti (1963) described as a vital growth component, thus destroying the cell. Their experimental evidence was that trypsin-isolated cells failed to survive in synthetic media, but grew well when NGF was added. The evidence presented above demonstrates that ganglion cells prosper in Eagle's solution or Medium 199 when untrypsinized. We therefore incubated ganglia in plasma or Eagle's solution containing trypsin $1-10^{-2}$ mg/ml for 24 h. A very characteristic destruction was observed: Figs. 17 and 18 illustrate the 'woolly' appearance which is followed by disintegration.
In some experiments on collateral reinnervation in the somatic motor nervous system (Hoffman, 1952) heparin was shown to inhibit collateral sprouting of motor axons. It seemed desirable to examine the effect of heparin on neuroblasts sprouting neurites. Ganglia were explanted on to gel discs in plasma or Eagle's solution containing 5–250 i.u. of heparin/ml. In concentrations from 25 to 250 units/ml lysis was observed, similar in character to that induced by antiserum, illustrated in Fig. 19.

DISCUSSION

Two quite different actions of NGF antisera on neuroblasts have been demonstrated here. In the presence of serum or plasma (or some component of these) lysis occurs. In the absence of serum proteins, in defined synthetic media, lysis does not result; however, the characteristic differentiated neuroblasts seen in control cultures are absent or severely reduced in numbers. Instead of these, a stream of tiny 'embryonic' cells emerges from the ganglia. Under these conditions the cell population both inside the ganglionic mass, and freely migrating on the gel surface, appears to be a less differentiated one than that originally explanted. There are fewer large neuroblasts, and more embryonic cells after incubation than are present at the time of explantation. Most of the cells seen after incubation in synthetic medium containing NGF-antiserum resemble those seen in cultures of very early central nervous system, or those seen in ganglionic masses soon after their first appearance in the embryo.

When examined in stained sections of fixed cultures, either by light or electron microscopy, they have the characteristic appearance of the 'undifferentiated' cells described in ganglia of early (3–4-day) chick embryos by Pannese (1968). They are recognizable by their round nuclei, conspicuous nucleoli, sparse basophilic cytoplasm, distributed in a thin perinuclear rim and packed with ribosomes. They bear no similarity to mature or immature satellite cells. Thus, antiserum under these conditions inhibits differentiation; it may even act to initiate dedifferentiation of already differentiated neuroblasts. In this context it is interesting to note that the cell population emerging from explanted ganglia in plasma or serum is a more differentiated one than in Eagle's solution: there is a higher proportion of differentiated neuroblasts and a lower proportion of embryonic cells in plasma or serum. It is possible that this can be explained by assuming a moderate concentration of NGF in the culture-media of animal origin (plasma or serum) enabling cells which are undifferentiated when they emerge in vitro to pick up and bind NGF from the medium, initiating their differentiation. The cells which are seen to differentiate while isolated on the gel surface in Eagle's solution must be assumed to have carried bound NGF with them from the explant. Any free NGF in the ganglionic tissue fluid at the time of explantation would be diluted in excess of $10^4 \times$ by diffusion in the medium.
Thus any cells moving about on the gel would encounter insignificant concentrations of NGF. The differentiation of some neuroblasts under these conditions, and the possibility of blocking or reversing this with antibody, further supports the hypothesis that NGF may become firmly bound to the cell surface, and that this attachment precipitates the processes of differentiation.

Possibly lysis occurs when antibody attaches to the firmly bound NGF on the cell membrane, together with some other protein cofactor, which enables either a firm attachment of antibody to the cell membrane, followed by puncture, or a rupture of the antigen-membrane complex, resulting in irreversible membrane damage. The action of antibody in arresting and reversing differentiation in the absence of cofactors might similarly be attributed either to its attaching more firmly to NGF than NGF is bound to the cell membrane, possibly followed by detachment of the antibody-NGF complex from the cell, or alternatively the antibody might attach to the NGF, which remains bound to the cell membrane. In this latter case the steric distortion which the antibody would induce in NGF might modify any effect within the neuroblast due to NGF action.

Since trypsin has been shown to be quite toxic to neuroblasts in relatively low concentrations, the inability of trypsin-isolated neuroblasts to grow in synthetic media (Levi-Montalcini & Angeletti, 1963) is readily understandable. Even though the conditions of growth in trypsin-containing medium described here are not strictly comparable with those used by the previous experimenters, it should be pointed out that the concentrations used here were far lower than theirs, and, since these low concentrations proved so extremely toxic, it is possible that brief treatment at higher concentrations would damage the cells. The revival of these cells by added NGF is less readily explained: trypsin may specifically injure some membrane structure which requires NGF in order to regenerate.

It is obvious that different groups of neurons have different sensitivity to lysis by NGF-antibody. Even in the newborn animal, as shown by Vogt (1964) and Zaimis et al. (1965) the prevertebral ganglia are much less sensitive than the paravertebral. It is further clear that part of the paravertebral population loses its sensitivity in the adult phase where only 60% or less of cells can be destroyed by antibody. It is significant that NGF antibody destroys only those cells in the newborn mammal which are capable of responding to added NGF: the sympathetic neurons. The sensory neurons, sensitive in the embryonic phase to NGF, lose this sensitivity later, and are likewise resistant to NGF antibody. Possibly this changing sensitivity is due to some cell populations reaching a stage of maturation at which any membrane-bound NGF is lost, and the cell is then no longer a target for antibody attachment and lysis.
SUMMARY

1. When chick sensory ganglia are explanted in plasma, serum or synthetic media such as Eagle’s solution or Medium 199 on an acrylamide gel surface, considerable migration of the cell population occurs over the gel surface.

2. In the presence of antiserum against the ‘Nerve Growth Factor’ (NGF) neuroblasts growing in plasma or serum are lysed, resulting in residual cell ‘ghosts’.

3. In synthetic medium no such lysis is observed, instead differentiation of neuroblasts is apparently inhibited, and an embryonic cell population results.

4. Lysis is apparently achieved in the presence of some serum cofactor or cofactors, while in the absence of such cofactors, the more typical antagonism of NGF by its antibody—the prevention of differentiation—is revealed.

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REFERENCES


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