In vitro studies of limb regeneration in adult Diemictylus viridescens: Neural dependence of blastema cells for growth and differentiation

By MORTON GLOBUS¹ AND RICHARD A. LIVERSAGE

From the Ramsay Wright Zoological Laboratories,
University of Toronto

SUMMARY

Explants of 99 adult newt forelimb blastemata (21- to 24-day regenerates) were cultured, with and without implanted dorsal root ganglia, in modified Parker's medium (CMRL-1415) for periods of 72-144 h. Growth and differentiation of the cultured blastemata were compared with ganglionated and non-ganglionated controls fixed at the start of the culture period.

The results of these experiments establish that implanted spinal ganglia are able to sustain growth and differentiation of forelimb blastemata in vitro: active proliferation amongst the blastema cells was found to be correlated with the presence of an implanted ganglion. In addition, the blastema cells exhibited a differential growth response which was most pronounced when the ganglion was eccentrically implanted 2-3 days before explantation of the limb regenerate.

These results suggest that a causal relationship exists between the position of the implanted ganglion and the localization of growth within the blastema. The nerve influence, believed to be mediated by a chemical factor(s), was localized in the region of the implanted neurons, indicating that a close association between the nerves and the responding blastema cells is essential for normal growth. The importance of the physical presence of nerves for the cultivation of blastemata in vitro is emphasized.

INTRODUCTION

Limb regeneration in urodeles is a nerve-dependent phenomenon (see reviews by Singer, 1952, 1960; Thornton, 1968). Singer (1952) has shown that all neurons, irrespective of origin (motor, sensory and autonomic), are qualitatively capable of supporting limb regeneration; however, growth of the blastema occurs only when the volume of neuroplasm in the regeneration area exceeds a threshold quantitative level (Rzehak & Singer, 1966; Singer, Rzehak & Maier, 1967). Blastema cell proliferation, which is most prevalent during the cone stage of regeneration (Chalkley, 1959), is greatly influenced by the presence of nerves, and a critical mass of cells does not accumulate in a denervated limb (Singer & Craven, 1948). Moreover, the trophic influence of neurons in larval and adult urodele regeneration appears to be wholly local and does not require interconnections with the central nervous system (Liversage, 1959, 1962).

¹ Author's address: Department of Biology, University of Waterloo, Waterloo, Ontario, Canada.
tion of urodele forelimb proceeds when segments of brachial nerve, containing only axons, Schwann cells and connective tissue cells (Wallace, 1972; Wallace & Wallace, 1973) or isolated sensory ganglia (Kamrin & Singer, 1959) are implanted into denervated limbs.

Although nerves are essential in urodele regeneration, the precise nature of their influence remains elusive. Recently, Globus (1970) and Globus & Liversage (1975) cultured larval *Ambystoma* tail blastemata with their epidermal covering and integral nerve supply. These innervated young tail blastemata underwent growth and differentiation in vitro resulting in a rod of cartilage ventral to the regenerating spinal cord. In those experiments we assumed that neural dependence of the blastema for growth and differentiation would be retained in vitro. In order to test our assumption, we would like to have compared the behaviour of innervated with denervated regenerates in culture, but the tail explant, with its 'built-in' nerves, paradoxically proved to be unsuitable because denervation results in the consequent destruction of the regenerate. On the contrary, a limb blastema explant would be essentially nerveless because explantation separates the peripheral nerve processes from their cell bodies, thus effecting a denervation. Innervated limb regenerates could be prepared by implanting dorsal root ganglia into the blastemata prior to explantation. The current work was, therefore, undertaken to test our original assumption and to study, in vitro, nerve–blastema interactions in a cause and effect relationship.

**MATERIALS AND METHODS**

Adult newts (*Diemictylus (Notophthalmus) viridescens*) obtained from central Massachusetts were kept in dechlorinated tap water and fed lean ground beef twice weekly. Medium-sized animals of both sexes (1.8-2.2 g body weight) were anesthetized in MS-222 (1:1000, Sandoz) and amputated through the mid-humerus of the forelimb. Forelimbs regenerated at 20 (± 1) °C in water which was changed daily to reduce bacterial contamination introduced by excreta.

**Spinal ganglion implantation**

The 3rd and 4th brachial spinal ganglia (neurons innervating forelimb) and the 16th and 17th crural ganglia (innervating hind limb) were excised and implanted homoplastically into the regenerating forelimb of host animals either 10 or 19 days post-amputation (Fig. 1). Initially, all regenerates received either a distal or proximal ganglion implant so that they were equally innervated until culturing. Distal implantation of a donor ganglion was done as follows: (1) a small slit was made in the skin of the host limb a few millimeters proximal to the original level of amputation; (2) the subcutaneous tissues were tunnelled distally, using fine watchmaker’s forceps; then (3) a donor ganglion was pushed through the tunnel until it came to rest under the covering epidermis of the blastema. Proximal implantation was achieved by placing the ganglion about
Fig. 1. Graphic representation of ganglionation and explantation procedures. ●, Legend ganglion.
proximal to the intended level of amputation at explantation. The latter approach provided a regenerate with a donor ganglion up to the time of culture, which was later excluded from the explant (see Fig. 1). Moreover, host limb nerves (brachial) were not transected since explantation of the regenerate ultimately separated the nerve processes from their cell bodies, thus effecting a denervation.

**Explantation**

Early cone-stage regenerates of anesthetized animals were excised several millimeters proximal to the original amputation level and immersed in a 1% chloramine antiseptic solution (sodium hypochlorite) for 90 sec. Exposure of limbs to this solution resulted in 95–100% sterility of the cultures. The excised regenerates (including 1 mm of the stump) were then rinsed in culture medium, transferred to a stainless-steel grid in a 35 x 10 mm plastic Petri dish, and cultured at the air-medium interface.

The cone-stage regenerates were explanted into nutrient medium with and without an implanted ganglion. The culture medium1 (CMRL-1415, Healy & Parker, 1966) was modified to amphibian salt concentration (225 ± 5 m-osmoles) and supplemented with 10% whole egg ultra-filtrate (GIBCO, New York), 8% fetal calf serum, 0.04 units of bovine crystalline insulin, 100 units of penicillin G potassium, and 50 μg streptomycin sulphate per ml of medium (latter three products from Connaught Medical Research Laboratories Ltd., Toronto). The addition of insulin (Vethamany, 1970; Vethamany-Globus & Liversage, 1973a, b) proved to be essential for the maintenance, growth and differentiation of the explants. Whole egg ultra-filtrate was utilized rather than chick embryo extract in order to have a medium exclusive of nerve tissue extracts. Cultures were incubated at 20 (± 0.5) °C, gassed daily with 5% CO₂ in air and maintained at a pH of 7.2–7.4. The medium was changed every 48 h and explants were cultured for various periods up to six days. At explantation, one half of the cases (both ganglionated and non-ganglionated time-zero controls) were fixed for histological examination; these served as a reference for the stage of regeneration at which the explantations were performed. The maintenance, proliferation and differentiation of all regenerates in vitro were assessed by comparing cultured pieces with control regenerates.

**Histology**

Limb regenerates were fixed in aqueous Bouin’s fluid and decalcified, when necessary, in Jenkins’ solution. They were embedded in paraffin, serially sectioned in a longitudinal plane at 8 μm, routinely stained with Delafield’s hematoxylin and counterstained with orange G-eosin. The presence of cartilage matrix was further verified using Alcian blue (pH 1.0) which indicates the presence of sulphated mucopolysaccharides (Quintarelli & Dellovo, 1965).

1 The culture medium and the culture assembly were developed in collaboration with Dr Swani Vethamany-Globus (Vethamany, 1970).
**Table 1. Distribution of culture groups**

<table>
<thead>
<tr>
<th>Morphological stage of regenerate at time of explantation</th>
<th>Early cone</th>
<th>Midcone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without stump</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ganglionated</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>Non-ganglionated</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>With stump</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ganglionated</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Non-ganglionated</td>
<td>8</td>
<td>33</td>
</tr>
</tbody>
</table>

**RESULTS**

This study is based on observations of 99 explanted forelimb regenerates (with and without ganglion implants), fixed after a culture period of 3–6 days, and 46 time-zero control regenerates, fixed at the start of the culture period. Table 1 illustrates the distribution of the experiments and the pertinent results are represented graphically in Fig. 2.

**Innervation**

Until explantation all ganglionated blastemata were also innervated by the intact peripheral nerves of the host. Likewise, the control ganglionated blastemata were normally innervated and also contained implanted neurons until fixation. Thus, in explanted ganglionated blastemata, the distally implanted spinal ganglia remained the sole nerve source serving the cultured regenerates.
In this regard, the non-ganglionated, cultured regenerates were effectively nerveless, because their proximally implanted ganglion was excluded from the explant (Fig. 1).

Although an attempt was made to implant a ganglion (at 10 or 19 days post-amputation) in an eccentric location, cell and tissue movements in the regeneration area tended to displace the ganglion. Therefore, the influence of an implanted ganglion will be described relative to its definitive location within the explant. These were: (1) eccentric within the blastema; (2) central within the blastema; and (3) lateral to the distal tip of the stump bone.

**Differential blastema cell distribution**

Spinal ganglia implanted into early blastemata 19 days post-amputation had a marked influence on the blastema cell population of cultured early cone-stage explants (Fig. 3). The source of the ganglia (whether from regenerating or non-regenerating donor animals) did not seem to enhance or otherwise influence the quality of the results. In ganglionated explants cultured for 4 days (Fig. 3) the greatest density in the population of blastema cells is seen near the implanted ganglion; the cells were contiguous to one another (little intercellular space) and they exhibited numerous mitotic figures. This effect was most pronounced when the ganglion was eccentrically positioned within the explant. In areas more removed from the ganglion, the blastema cell density was sparser, there were fewer mitotic figures and there was considerably more intercellular space. Increased cell density near the ganglion was unique to these cultured blastemata; early cone-stage time-zero controls (Fig. 4) showed a homogeneous distribution of overtly undifferentiated blastema cells in both ganglionated and non-ganglionated blastemata. Although mitotic figures were evident, there appeared to be no particular concentration of them in any region of the blastema. Non-ganglionated explants, cultured for 4 days, exhibited a relatively homogeneous distribution of blastema cells (Fig. 5) similar to that found in the time-zero controls. Although mitotic figures were observed, this activity was not more
Nerve dependent regeneration in vitro

conspicuous in any one region than another and distinct areas of high cell density were not observed. Similar observations were recorded in a parallel series of ganglionated and non-ganglionated explants, in which only the blastema was cultured (no stump – Fig. 1, bottom). An increase in the blastema cell density, unique to the cultured innervated regenerates, was seen in the vicinity of the ganglion (Fig. 6), and numerous mitotic figures were observed (Fig. 7). The non-ganglionated explants (Fig. 8) and time-zero controls did not exhibit areas of high cell density.

Contrary to the above, distinct areas of high cell density were not observed in explants with a centrally positioned ganglion implanted at 19 days post-amputation. Although numerous mitotic figures were present among blastema cells around the ganglion, the cellular density appeared to be uniformly increased throughout the regenerate (Fig. 9). A similar result was obtained when a ganglion was implanted into a regenerating limb 10 days post-amputation (approximately 12 days before explantation). Under these circumstances, the density of blastema cells was not increased exclusively in the vicinity of the ganglion, and, again, a more or less uniform increase in blastema cell proliferation was observed. This is in sharp contrast to the localized effects observed following eccentric ganglion implantation at 19 days post-amputation.

After 6 days in vitro, ganglion cells showed no signs of degeneration; they retained a distinct nuclear membrane and prominent nucleoli (Fig. 10). However, the associated cut peripheral nerve stumps showed considerable degeneration. Presumably, most of the degenerating axons were motor root fibers that had been severed when the ganglionated explant was excised (see also Kamrin & Singer, 1959).

An interesting phenomenon was repeatedly observed in cultured blastemata in relation to a ganglion implanted into a 10-day blastema and its effects on bone dedifferentiation. The ganglion was usually found embedded in the

**Figures 6-8**

Longitudinal sections

Fig. 6. Ganglionated forelimb blastema explant (no stump) cultured for 6 days, showing a dense population of blastema cells (db) near the eccentrically implanted ganglion (g) and a sparser population of blastema cells (sb) in areas more removed from the ganglion.

Fig. 7. A ganglionated blastema explant (stumpless), cultured for 6 days, showing mitotic figures (arrows) among blastema cells distributed in close proximity to the implanted ganglionic neurons. The neurons are not seen in the plane of view shown. In (A) blastema cells are in prophase (p) and metaphase (m), and in (B) two blastema cells are seen in anaphase.

Fig. 8. A non-ganglionated blastema explant (stumpless), cultured for 6 days, exhibiting a relatively homogeneous distribution of blastema cells (b) not dissimilar to that of time-zero controls. Note that distinct areas of high cell density cannot be discerned.
Nerve dependent regeneration in vitro

Blastema tissue at 22 days post-amputation; however, in some cases it remained in a position just lateral to the distal tip of the humerus. This resulted in more extensive dedifferentiation of the humerus on the side adjacent to the ganglion (Fig. 11) where osteoclastic activity was more pronounced. In addition, an accumulation of blastema cells was observed between the ganglion and the disto-lateral tip of the bone. This effect was observed only in cultured blastemata innervated solely by eccentrically positioned donor ganglia (in absence of normal innervation). When the ganglion resided either immediately distal to the humerus or embedded even more distally among the blastema cells, dedifferentiation appeared to proceed in a normal (unbiased) disto-proximal direction, resulting in an accumulation of blastema cells distal to the bone. Control ganglionated regenerates consistently displayed a normal disto-proximal dedifferentiation (Fig. 4), presumably, because they were innervated by host peripheral nerve fibers in addition to a donor ganglion.

Differentiation in ganglionated explants

Ganglionated mid-cone stage blastemata cultured for periods of 4–6 days formed cartilage in vitro which was evident, in some cases, as a procartilage condensation, and in others as an advanced stage of cartilage differentiation (Fig. 12), distal to the stump bone(s). In the latter cases a dense matrix has been deposited around the cells, and distal to the cartilage blastema cells were aligned almost to the tip of the regenerate. The corresponding controls, fixed at the time of explantation, were composed primarily of overtly undifferentiated blastema cells (Fig. 13). In this series of experiments, ganglia were implanted at 19 days post-amputation (see Fig. 1) and amputations were performed either through the humerus or through the forearm (radius and ulna). Furthermore, the definitive position of the ganglion seemed to influence the distribution of the blastema cells and, perhaps, their subsequent differentiation. When a ganglion occupied a position in the blastema just distal to the radius (Fig. 14) the cartilage distal to the ulna toed-in toward the ganglion. Our results show that ganglionated

Figures 9-11

Fig. 9. A longitudinal section of a ganglionated blastema explant cultured for 6 days. This explant, having a centrally implanted ganglion (g), does not exhibit a distinct area(s) of high cell density. The cellular density was increased equally throughout the regenerate. The area enclosed by a rectangle is enlarged in Fig. 10.

Fig. 10. Higher magnification of a portion of Fig. 9 showing ganglion cells (g) with distinct nuclear membranes and prominent nucleoli. A blastema cell in telophase (t) is seen close to the ganglion.

Fig. 11. A longitudinal section of a ganglionated blastema explant (with stump), cultured for three days, showing more extensive dedifferentiation of bone on the side of the humerus (h) adjacent to the implanted ganglion (g). Note the biased osteoclastic activity (ost) on the innervated side. The arrows indicate the level of amputation.
Nerve dependent regeneration in vitro

early cone-stage blastema explants undergo a conspicuous increase in blastema cell density in the vicinity of the ganglion, whereas, when ganglionated blastemata are explanted at the mid-cone stage, blastema cell condensation and cartilage differentiation were observed.

DISCUSSION

The present results establish that implanted spinal ganglia are able to sustain growth in the adult newt forelimb blastema in vitro, and also suggest that a causal relationship exists between the position of the ganglion and the localization of growth within the blastema. Support for this interpretation is provided by experiments of Singer, Ray & Peadon (1964) which showed that growth in the early blastema occurred mainly from the ventral and posterior quadrants of the blastema where the major nerve trunks of the limb are located. When the nerve trunks were shifted to the dorsal and anterior regions of the blastema, the cellular contribution to growth from the new regions was increased. Our results and those above suggest that the growth potential in different areas of the early blastema must be equivalent and in this context the blastema was perhaps aptly described by Butler & Puckett (1940) as an 'equipotential aggregate of cells'.

Under the present experimental conditions (ganglionated blastemata) localized growth, observed near the implanted neurons, was most pronounced when the ganglion was eccentrically implanted 19 days post-amputation. In such cases fiber regeneration was restricted to a period of 2 or 3 days preceding the beginning of culture and during this short period was probably limited to the region adjacent to the ganglion, resulting in a localized stimulus to blastema cell proliferation. In some instances the ganglion was centrally positioned in the explant so that the blastema cells were equally disposed to its influence. In these cases it might be inferred that the ganglion's sphere of influence was

FIGURES 12-14

Longitudinal sections. Arrows indicate the level of amputation.

Fig. 12. A ganglionated mid-cone stage blastema explant (with stump), cultured for 4 days, showing advanced cartilage differentiation distal to the radius (r) and ulna (u). A dense matrix has been deposited around the chondrocytes and the cells have undergone a typical flattening. The implanted ganglionic neurons cannot be seen in this section but are located in adjacent sections in the position marked by a g, indicating ganglion.

Fig. 13. A mid-cone stage forelimb blastema showing the extent of regeneration in a ganglionated time-zero control. Procartilage alignment is scarcely evident. This limb was amputated through the radius (r) and ulna (u). The ganglion (more lateral) is not seen in this median-longitudinal section.

Fig. 14. A ganglionated blastema explant, cultured for 4 days, showing cartilage (c) distal to the ulna (u) toed-in toward the ganglion (g). The ganglion is positioned just distal to the radius (r).
symmetrical and therefore a differential response of the cells was not observed. Rather, the cell density was increased equally throughout the regenerate. Similarly, when a ganglion was implanted into a regenerating limb approximately 12 days prior to explantation (10 days post-amputation), the influence of the ganglion was manifested in a general increase in blastema cell proliferation. At the time of implantation, mesenchymatous cells had not accumulated to form a blastema. Presumably, regenerating nerve fibers originating from the neurons of the implanted ganglion (see Kamrin & Singer, 1959) subsequently innervated blastema cells as they accumulated distally. In these cases fiber regeneration could proceed in vivo for 12 days following ganglion implantation and preceding the beginning of culture. It was therefore assumed that the blastema explants, when subsequently cultured, were uniformly innervated by nerve fibers from the implanted ganglion. Consequently localized growth, as reflected in a differential blastema cell density, was not observed.

In addition to increasing the mitotic activity of blastema cells adjacent to the ganglion, an implanted ganglion may conceivably influence their mobilization (see also Schotte & Butler, 1941, 1944; Butler & Schotte, 1949; Singer et al. 1964). Blastema cells were seen in some ganglionated explants to orient themselves with their long axes toward the ganglion, suggesting that cell migration could have contributed to the increased population of cells found in the vicinity of the ganglion of cultured blastemata.

The neurotrophic agent responsible for these effects on the regeneration blastema has not been identified, although it is generally considered chemical in nature and released from the axonal endings of the neurons where it exerts its influence on the responding tissues (Singer, 1974). Our results suggest a differential growth response within the blastema which is related to the proximity of the cells to the implanted ganglion. Thus, it appears that the nerve influence does not extend far from the implanted neurons; rather it exerts its influence predominantly on the closest blastema cells which is consistent with the findings of Singer et al. (1964). The proposed chemical agent of the nerve must therefore be effective only where its concentration is greatest, namely in proximity to the neurons. Equally acceptable are the possibilities that the trophic agent does not diffuse far from its site of release, and/or that it is a labile substance which is rapidly inactivated and must be constantly renewed, and/or that it can only be transmitted when there is immediate contact between the nerve process and the responding cell. The latter possibility is also suggested by ultrastructural studies (Salpeter, 1965), which show that the apposing membranes of nerve and mesenchyme cells are separated by a narrow gap of 100–200 Å, thus permitting the trophic influence to be mediated by a short-lived chemical substance. In an actively growing blastema, utilization or inactivation of a trophic substance would necessitate its constant renewal. Indeed, Kamrin & Singer (1959) found that living ganglion cells implanted into adult newt blastemata elicit regeneration in an otherwise denervated forelimb, whereas
freezing or heating of the ganglion immediately destroyed the effectiveness of
the ganglion as a growth stimulus. They concluded that a continuous flow of
the unknown neuronal factor is required for growth.

A search for nerve-dependent biochemical changes in the regenerating newt
limb blastema (Dresden, 1969; Lebowitz & Singer, 1970; Singer & Caston,
1972) revealed a time-dependent loss in the capacity of the regenerate to
incorporate radioactive precursors into DNA, RNA and protein consequent to
denervation. Recently, it was shown that the protein synthetic capacity can be
partially or even totally recovered when crude nerve homogenates, brain
synaptosomal concentrates or soluble brain protein extracts are infused into
the regenerate (Singer, 1974). Although it has been possible to stimulate
macromolecular synthesis, attempts to obtain an increase in the blastema mass
by infusing spinal cord or brain extracts into denervated stumps (Deck, 1971)
or into denervated early regenerate buds (Singer, 1974) have not been successful.
Similarly, when denervated early forelimb regenerates of Ambystoma opacum
larvae were cultured in medium containing spinal cord or brain tissue extract
(changed daily), growth of the blastema cell mass was not observed (Globus,
unpublished results). The current and other work cited above emphasize that
an intimate relationship between nerve cells and responding blastema cells may
be necessary for regeneration to ensue.

Although the importance of nerves in the early stages of regeneration is widely
accepted, their role in the later stages (including differentiation) remains un-
clear (Schotte & Butler, 1941, 1944; Butler & Schotte, 1949). These authors
found that nerves are essential for the induction and accumulation phases, and
reported that ‘thereafter the larval blastema becomes emancipated from neural
influences’. In the adult newt, Singer & Craven (1948) found that limb dener-
vation before the 13th day of regeneration (at 25 °C) suppressed further increases
in mass and length of the regenerate and also blocked mitotic activity. There-
after, limited increases in the length and abnormal bone differentiation occurred
in the absence of nerves; however, any further increase in mass was effectively
blocked. When denervation was performed after blastema formation (13 days
of regeneration) histogenesis and morphogenesis were found to be more inde-
pendent of nerves.

Nonetheless, a previous history of growth of the regenerate in the presence of
an adequate nerve supply appears to be essential for its subsequent emancipation
from neural influence (Singer, 1952). Another point to be considered is that the
density of blastema cells, which is influenced by nerves, may have a role in
blastema cell differentiation. In normally regenerating limb blastemata, localized
cellular condensations precede tissue differentiation, whereas in non-differenti-
ating (denervated) blastemata such condensations do not occur. In our studies
a conspicuously dense population of blastema cells accumulated around the
implanted ganglion, and subsequent orientation of procartilage formations
favoured the eccentrically implanted neurons. In addition to the established

Nerve dependent regeneration in vitro
role of nerves in the initiation, accumulation and proliferation phases of regeneration, they also appear to influence the density and distribution of the blastema cells and, thereby, determine the form and orientation of the resulting cartilage. Thornton (1959) suggested that nerves may ‘influence the cellular density or the cellular mass of the blastema, thus affecting intercellular behaviour critical to the processes of determination’. On the basis of these data, it seems reasonable to conclude that nerves have a role in blastema cell differentiation, albeit indirectly, by influencing the preceding events, namely, proliferation and cell condensation.

We wish to express our appreciation to Dr Swani Vethamany-Globus for her valuable suggestions and discussions throughout the course of this work and in preparation of the manuscript. We also wish to express our thanks to Mr George Healy and Dr R. G. Romans (Connaught Medical Research Laboratories, Toronto, Ontario) for supplying medium and insulin, respectively. This paper represents a portion of a Ph.D. dissertation submitted (by M.G.) to the Graduate Department of Zoology, University of Toronto. This investigation was supported by fellowships from the University of Toronto, Province of Ontario and National Research Council of Canada (to M.G.) and by grant no. A-1208 from the National Research Council of Canada (to R.A.L.).

REFERENCES


Nerve dependent regeneration in vitro


(Received 22 July 1974)