Differentiation in vitro of innervated tail regenerates in larval *Ambystoma*

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**Summary**

Larval *Ambystoma* tail regenerates, innervated by the intact spinal cord, were cultured with their epidermal covering in modified Parker’s medium (CMRL-1415) and the morphological integrity of the explanted regenerates was maintained with consistent success. The results show that the larval urodele tail blastema is capable of undergoing growth and differentiation when isolated in vitro and regeneration follows the normal in vivo pattern. A diffuse aggregate of blastema cells, which extended caudally from the cut end of the notochord at the time of explantation, underwent differentiation in vitro in the presence of the regenerating spinal cord. The importance of the nerve in regeneration is discussed in relation to the in vitro system.

**Introduction**

It is well established that regeneration of the urodele limb and tail is a nerve-dependent phenomenon (see reviews by Singer, 1952, 1960; Thornton, 1968). Singer has shown that all nerve fibers are qualitatively capable of supporting limb regeneration and stimulating growth of the regeneration blastema, however, growth occurs only when the number of nerve fibers in the regeneration area exceeds a threshold quantitative level (Singer, 1952). It is further believed that the neural contribution to growth of the regenerate is chemical in nature (Schotte & Butler, 1941; Singer, 1965, 1974). Regeneration of the urodele tail is also markedly influenced by nervous tissue. Tail regeneration is effectively suppressed (Godlewski, 1904; Goldfarb, 1909; Locatelli, 1929; Holtzer, Holtzer & Avery, 1955) following destruction or extirpation of the spinal cord subjacent to the wound surface of the tail; spinal ganglia alone are not adequate to support tail regeneration (Holtzer, 1956).

Although the nerves play an essential role in regeneration, the precise nature of their influence remains to be elucidated. The primary objectives of this work were to develop an in vitro system suitable for studies of the neural control of regeneration, and to determine the capabilities of the larval urodele tail blastema to grow and differentiate in vitro in the presence of nerves. This work was initiated with the underlying assumption that proliferation and subsequent

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differentiation of explanted blastemata are dependent upon the presence of nervous tissue (see also Globus, 1970; Globus & Liversage, 1975). The tail regenerate was considered (rather than the limb) as an ideal explant because of its integral innervation (spinal cord, spinal and autonomic ganglia). Anuran tails have been cultured by Weber (1962) and by Shaffer (1963) in relation to studies on metamorphosis of *Xenopus* tails. However, previous attempts to culture undifferentiated urodele limb blastemata have been met with limited success (Fimian, 1959; Stocum, 1968); a factor common to their attempts was that the explants lacked innervation.

**MATERIALS AND METHODS**

The larval urodeles (*Ambystoma opacum* and *Ambystoma maculatum*) used in this study were obtained in the pre-hatching stage from Tennessee and Massachusetts, U.S.A., respectively. Young larvae were reared in the laboratory, maintained in dechlorinated tap water at 20 ± 1 °C, and fed with *Daphnia* and *Tubifex* worms. Animals were deprived of food for 4 days prior to culturing in order to reduce bacterial contamination introduced into the water by way of excreta. Larvae measuring 27–33 mm in length were anesthetized in an aqueous solution of 1:5000 MS-222 (tricaine methane-sulfonate, Sandoz Chemical Co.). The tails were amputated midway between the cloaca and the tail tip and allowed to regenerate for 7 or 8 days at 20 ± 1 °C before the blastema was explanted. Following 7 days of *in situ* tail regeneration, the experimental animals were anesthetized, the regenerate (including stump) was excised, and immersed in an antiseptic solution of 1% chloramine (sodium hypochlorite) for 45 sec. The young tail blastema (including approximately 1 mm of the stump) was then rinsed in culture medium, transferred to a stainless-steel grid in a 35 x 10 mm Petri dish and cultured at the air-medium interface.

The culture medium¹ (CMRL-1415, Healy & Parker, 1966) was modified to amphibian salt concentrations (225 m-osmoles) and supplemented with 10% 12- to 14-day chick embryo extract, 8% fetal calf serum (Grand Island Biological Co., New York), 0.04 units of insulin, 100 units of penicillin G potassium, and 50 μg of streptomycin sulphate per ml of medium (latter three products from Connaught Medical Research Laboratories Ltd., Toronto). The addition of insulin (Vethamany, 1970) proved to be essential for the maintenance, growth and differentiation of the explants.

The 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 replaced every 48 h and the explants were cultured for 2–4 days. Corresponding time-zero controls were fixed when the experimental blastemata were explanted. These controls served as a reference for the stage of regeneration at which explantation of the blastema was performed. The maintenance, proliferation and further differen-

¹ The culture medium and the culture assembly were developed in collaboration with Dr Swani Vethamany-Globus (see Vethamany, 1970).
Table 1. *Differentiation of vertebral cartilage in larval urodele tail regenerates, in vitro*

<table>
<thead>
<tr>
<th>Series ...</th>
<th>Species ...</th>
<th>I. A. opacum</th>
<th>II. A. opacum</th>
<th>III. A. opacum</th>
<th>IV. A. maculatum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time-zero controls</td>
<td>Days of regeneration <em>in vivo</em></td>
<td>8</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Number of controls</td>
<td>22</td>
<td>20</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>Explanted tail regenerates</td>
<td>Days of regeneration <em>in vivo</em></td>
<td>8</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Days of regeneration <em>in vitro</em></td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Number of explants examined for cartilage differentiation</td>
<td>22</td>
<td>20</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>% exhibiting advanced differentiation</td>
<td>77</td>
<td>85</td>
<td>79</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>% exhibiting beginning differentiation</td>
<td>23</td>
<td>10</td>
<td>14</td>
<td>15</td>
</tr>
</tbody>
</table>

Differentiation of all explants *in vitro* were assessed by comparing the cultured pieces with control regenerates, fixed at the beginning of the culture period.

The tail regenerates were fixed in aqueous Bouin’s fluid, embedded in paraffin, sectioned serially at 8 μm in a sagittal plane, and stained with Delafield’s hematoxylin and orange G-eosin. The presence of cartilage matrix was further verified using the Alcian blue (pH 1.0) staining procedure, which indicates the presence of sulphated mucopolysaccharides in the matrix of cartilage (Quintarelli & Dellovo, 1965).

**RESULTS**

The results of this study were based on detailed observations of 69 explanted tail regenerates and 69 time-zero controls. Table 1 illustrates the results and distribution of the experiments. At approximately 7–8 days post-amputation (the time at which blastema explantations were made), blastema cells are arranged so that some surround the spinal cord but the bulk of the cell population lies ventral to it, forming a diffuse cell aggregate (Fig. 1) which extends caudally from the cut surface of the notochord (see also Holtzer, 1956). In larval urodeles, the notochord does not regenerate; in its place a series of segmental cartilages are formed (Holtzer *et al.* 1955). As a result, the junction between the cut surface of the notochord and the proximal border of the blastema cell aggregate serves as a permanent marker of the original amputation plane.

The salient feature exhibited by the explants is the considerable degree of cartilage differentiation (Table 1) achieved in culture (Fig. 2). The regenerating cartilage which is contiguous with the notochord and lies adjacent to the motor (ventral) surface of the spinal cord, acquired the form of a rod which tapers to a point just proximal to the distal tip of the spinal cord. The rod is composed, principally, of procartilage cells with blastema cells circumjacent to it, whereas
the terminal tip of the rod is composed of loosely arranged blastema cells, many of which are undergoing mitosis. The procartilage cells are surrounded by a basophilic matrix; however, at the time of explantation, neither blastema cell condensation nor matrix deposition was detected in time-zero controls (see Fig. 1). Mitotic figures were observed among blastema cells and procartilage cells but more rarely among the latter; blastema cells in mitosis were seen circumjacent to the differentiating rod of cartilage, at the distalmost tip of the rod and juxtaposed between the spinal cord and the forming cartilage. When the duration of the culture period was increased from 2 days to 3 or 4 days, cartilage differentiation was more advanced; the chondrocytes were surrounded by a dense matrix, and the cartilagenous rod became thickened in profile.

Epidermal wound healing of the proximal freshly cut surface of the explant also occurred in culture within 24 h after explantation. Frequently, mitotic figures were observed in the epidermis of the explants, especially in the thickened apical epidermal cap; however, mitotic activity was rarely seen in the epidermis covering the proximal wound surface.

Sarcoplasmic budding from the lateral stump muscle as observed in vivo, apparently continues in vitro. The cells were at first spindle-shaped, with an elongate, centrally placed nucleus and surrounded by a thin rim of cytoplasm (Fig. 3). But during the process of dedifferentiation or shortly after, these cells acquired a rounded shape and were seen to populate the region of the explant, lateral to the central axis and subjacent to the epidermis. Mitoses were frequently observed in this region.

The culture medium used in this investigation was favourable for the cultivation of ganglia (Fig. 4) and spinal cord (Fig. 5); frequently, mitotic figures were observed among the mesenchymal cells close to the ganglia. The regenerating spinal cord of the explant exhibited the typical morphology described in other in vivo studies (Butler & Ward, 1965) with an ependymal layer (cuboidal cells with spherical nuclei) lining the central canal, a peripheral layer of mantle cells,

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**Figures 1–3**

The arrows indicate the level of amputation

Fig. 1. Seven-day time-zero control showing the spatial relationships existing among the tissues of the regenerating larval tail. Note the diffuse blastema cell aggregate (ba) ventral to the spinal cord (sc); the terminal vesicle (tv) of spinal cord; notochord (n); epidermis (ep). Mid-sagittal section.

Fig. 2. Seven-day tail blastema explant cultured for 4 days showing the differentiation of vertebral cartilage (c) ventral to the spinal cord (sc). The matrix of cartilage is clearly evident and chondrocytes in the proximal region of the rod exhibit a typical flattening in a proximo-distal direction. Mid-sagittal section.

Fig. 3. Eight-day tail blastema explant cultured for 2 days, showing the liberation of a spindle-shaped mononucleate cell (arrow) from the muscle (m). Another cell is seen assuming a rounded shape while still associated with the muscle by a strand of cytoplasm (double arrow). Sagittal section, lateral to the central axis.
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and a terminal vesicle composed of a single layer of ependymal cells (columnar cells with oval nuclei). Considerable mitotic activity was observed in the ependymal layer (Fig. 5) throughout the length of the regenerating spinal cord of the explant. However, only a few mitotic figures were observed in the terminal bulb and mitoses in the extra-ependymal layers of the spinal cord were rarely seen. Explants cultured for two days had a terminal vesicle (Fig. 6) at the proximal freshly cut end of the spinal cord. This terminal vesicle, formed in vitro, was also composed of a single layer of columnar ependymal cells with oval nuclei.

DISCUSSION

In these experiments, young innervated larval tail regenerates were cultured in a nutrient medium and observations were made of the regenerative events that ensued over a period of up to 4 days. The results show that the diffuse aggregate of blastema cells that accumulated caudal to the cut end of the notochord at the time of explantation, underwent growth and differentiation in vitro. On the basis of the results, it has become apparent that an intact epidermal covering is essential in retaining the morphological integrity of the urodele regeneration blastema in culture; indeed, culturing of limb blastemata in the absence of an epidermal covering results in a loss of structural organization within the explant. A sheet of cells migrates from such explants (Fimian, 1959; Stocum, 1968) leaving only a central mass of mesenchymal cells, thereby resulting in a gradual loss of organization. Moreover, the addition of insulin to the medium was found to be necessary for the growth and differentiation of the larval tail explants. Working with adult Diemictylus, Vethamany (1970) and Vethamany-Globus & Liversage (1973a, b) showed that (1) insulin insufficiency results in an interference with normal adult limb and tail regeneration and (2) that regenerating adult newt tail blastemata require insulin for growth and differentiation in vitro. Our results (Globus, 1970; Globus & Liversage, 1975) further substantiate the requirement for insulin in regeneration.

Figures 4–6

Fig. 4. Tail blastema explant showing a typical spinal ganglion (A) composed of large neurons (n) with eccentrically positioned nuclei. A mitotic figure (indicated by an arrow) can be seen associated with the ganglion. (B) Ganglionic neurons (arrows) with distinct nuclear membranes and nucleoli. A mesenchymal cell in mitosis (m) is seen close to the neurons. Sagittal section, lateral to the central axis.

Fig. 5. Tail blastema explant showing considerable mitotic activity of ependymal cells (arrows) in the regenerating spinal cord. e, Ependymal layer; m, mantle layer; nc, neural canal. Mid-sagittal section.

Fig. 6. Eight-day tail blastema explant cultured for 2 days, showing a terminal vesicle (tv) of the spinal cord which has formed in vitro at the proximal (p) freshly cut end of the cord. Mid-sagittal section.
Butler and Schotte (1941, 1949) showed that after amputation the larval limb and its blastema regress in the absence of peripheral nerves. Similar results were obtained when larval cone-stage limb blastemata were cultured in the absence of nervous tissue (Globus, unpublished results). Also, Stocum (1968) found that the blastemal portion of larval *Ambystoma* cone explants ‘completely disappeared in 77.7% of the cases, leaving only the stump as a morphological entity’. The blastemal portion of his explants (nerveless) was maintained only after differentiation had begun and morphogenesis was well under way.

Since it has been clearly demonstrated (Singer, 1952, 1965) that the presence of nerves is essential for normal limb and tail regeneration *in vivo*, it is reasonable to assume that the neural dependence of blastema for growth and differentiation will be retained *in vitro*. Our observations show that a diffuse aggregate of overtly undifferentiated blastema cells undergoes differentiation in the presence of the spinal cord, giving credence to our assumption. However, to test this further, it would be necessary to compare the behaviour of innervated and denervated regenerates *in vitro*. Despite the advantage of ‘built-in’ nerves the tail explant paradoxically proved to be unsuitable for studies of denervated regenerates. Denervation of a tail explant is extremely difficult, if not impossible, because this type of manipulation results in the consequent destruction of the regenerate. In a related study, Globus (1970) and Globus & Liversage (1975) established that growth of an adult newt forelimb blastema could be sustained *in vitro* in the presence of an implanted dorsal root ganglion but the blastema cells failed to proliferate when cultured in the absence of an implanted ganglion. These findings further support our assumption and amplify the importance of nerves for the cultivation of blastemata *in vitro*.

Inasmuch as the spinal cord plays a very essential role in regeneration of the larval urodele tail (Holtzer, 1956), and also forms an integral part of the regenerate itself, the maintenance and viability of the spinal cord cells *in vitro* is of prime importance. The medium we employed maintained spinal ganglia and supported regeneration of the larval spinal cord; considerable mitotic activity was observed in the ependymal layer throughout the length of the regenerating spinal cord. Although comparisons between *in vivo* and *in vitro* observations should be viewed with caution, regeneration of the larval tail in this study appeared to proceed in a manner remarkably similar to that described by others *in situ*. We observed the release of mononucleate cells from the lateral stump muscle of the explanted tail. This phenomenon has indeed been previously reported *in vivo* by Holtzer (1956) and Thornton (1938) in their regeneration studies of larval tail and limb, respectively; however, this is the first instance where ‘sarcoplasmic budding’ has been reported *in vitro*.

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REFERENCES


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