Observations on proliferating sheath cells in the regenerating nerves of lizard

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Summary. The proliferation of sheath cells (Schwann and endoneurium) of growing nerves has been studied by autoradiography after ³H-thymidine administration to lizards (Anolis carolinensis and Lampropholis delicata) with regenerating tails. Schwann cells of regenerating nerves derive from the multiplication of resident cells within the growing nerves, but labelled Schwann cells derived from the regenerative blastema also appear to ensheath the new axons. Endoneurium cells of growing nerves derive from the blastema around the nerves more than from dividing and migrating cells of the original endoneurium. After 6-20 days post-injection the number of labelled sheath cells in the older proximal regenerated nerves increased due to local division and migration. In the proximal spinal ganglia which innervate the regenerating tail some satellite cells, but not neurons, took up ³H-thymidine and multiplied.

Key words: Lizard, Regenerating nerves, Sheath cells, Autoradiography

Introduction

In the regenerating tail of several species of lizards, the tissues most responsible for the rapid growth of the tail are represented by the axial cartilage spinal cord and muscles (Simpson, 1965; Cox, 1969; Alibardi and Sala, 1983). The morphogenesis of the regenerating scales, dermis, skeleton, spinal cord and peripheral nerves, follows a different pattern with respect to that observed during embryogenesis (Bellairs and Bryant, 1985; Alibardi, 1994a,b). The innervation of the new tail derives from the three most proximal spinal ganglia and spinal cord close to the regenerating tail (Terni, 1920, 1922). No nerves are produced by the new very simplified spinal cord encased in an uninterrupted cartilaginous skeleton (Hughes and New, 1959; Simpson, 1965; Cox, 1969; Alibardi and Sala, 1983; Bellairs and Bryant, 1985; Alibardi, 1994c).

Small and large axial nerves soon invade the

regenerating tail and reach the apex of the regenerating blastema, under the apical epidermis (Alibardi and Miolo, 1990). The above studies also reported that a certain number of ³H-thymidine-labelled cells were seen around the reactive axons entering the regenerating tail. Transected nerves soon invade the blastema (Hughes and New, 1959; Cox, 1969) and it is though the sheath cells (Schwann and endoneurial) proliferate from the transected nerves, descend along regenerating nerves, multiply and extend around the growing axons.

In the apical blastema, however, the regenerating nerves are seen as naked growing cones that closely contact blastematic and muscular cells (Alibardi and Miolo, 1990). Other fibroblastic-like cells in the regenerating tail may represent free Schwann cells which have migrated from transected nerves of the tail stump into the blastema. These cells may contribute, in a still unknown manner, to the cell composition of the regenerating blastema in lizard (Alibardi and Sala, 1983, 1988).

The detailed electron microscopic study of regenerating nerves, coupled with autoradiography after ³H-thymidine administration, may clarify the origin of ensheating cells. The present observations suggest that Schwann cells are derived from cell division within growing nerves and also from migration of blastematic cells into the regenerating nerves. The present study also shows that the new endoneurium appears mostly to be derived from the migration of cells from the blastema.

Materials and methods

Adults (males and females) of the lizards Anolis carolinensis and Lampropholis delicata, kept at 22-30 °C with a 12-hour photoperiod, were used in this study. The animals received, an injection of 10-15 μ Ci/g bw of ³H-thymidine (6-³H-thymidine, Amersham, s.a. 29 Ci/mmole) and were processed for autoradiography as previously described (Alibardi, 1994c,d).

From 5 animals (*L. delicata*) with normal tail, the sampling was done at 5 hours post-injection. Another 10 animals (5 *L. delicata* and 5 *A. carolinensis*) with three-week-old regenerating tails 3-5 mm long, were sampled at 4-5 hours post-injection (both in the regenerating and in the original tail). More sampling was done at 2 days

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(4 A. carolinensis), 4 days (4 A. carolinensis), 6 days (7 L. delicata), 12 days (4 A. carolinensis and 7 L. delicata), 20 days (6 L. delicata), and 25 days (3 A. carolinensis) post-injection.

The length of the regenerating tail in the samplings varied between 3-5 mm at three weeks of regeneration up to 25 mm at 46 days of regeneration (3 weeks + 25 days post-injection).

Pieces of skinned tail, 1-3 mm long, were fixed in 2.5% glutaraldehyde in Ringer at 4 $^{\circ}$ C, at pH 7.4-7.6 for 5-8 hours. After rinsing in Ringer fluid, the tissues were immersed in 1% OsO₄ for 2 hours, dehydrated and embedded in epon or araldite according to routine procedures.

After sectioning with an ultramicrotome, semithin or thin plastic sections were coated with Ilford K4 or L5 nuclear autoradiographical emulsion for variable periods (1-4 months). The samples were then developed with Kodak D19 and fixed with Ilford fixer. Semithin sections were lightly stained with 0.5% toluidine blue and studied and photographed with a light microscope. Thin sections were lightly stained with uranyl acetate and lead citrate, and observed with CX 100 or Hitachi 600 electron microscopes.

For quantification, labelled cells were counted out of 200-300 total cells in each sample (1-3 sections studied in the same animal), the mean of labelled cells was calculated and expressed as percentage of labelling $(\%L)\pm$ Standard Deviation (\pm SD). In the text, the number of animals studied is reported in parenthesis (N=).

Results

The number of labelled cells varied not only in relation to the post-injection time, but also according to the position along the nerves. We have considered as distal, naked or unmyelinated nerves within about 1 mm from the regenerating tail tip; medial, nerves between 2 and 5 mm (depending from of the actual length of the tail sample); proximal, nerves within 2 mm from the tail stump.

In the normal tail, no labelled cells were seen, aside in 2 out of 7 cases studied, where one occasional sheath cell was labelled.

In a regenerating tail 3-5 mm long, at 4-5 h postinjection, labelled cells were more commonly seen around the growing nerves than in the loose connective tissue or the regenerative blastema (Figs. 1, 2). At 4-5 hours post-injection, no labelled satellite cells of the spinal ganglia proximal to the regenerating tail were seen. However, both in *L. delicata* and *A. carolinensis*, labelled satellite cells were sometimes seen after 4 or 6 days from the injection ($6.28\pm1.67\%$; N=5; Fig. 3).

The observation of regrowing nerves from the tip of the regenerating tail till about 3 mm proximal, showed a high number of labelled cells at 4-5 hours post-injection surrounding or associated to nerves (Fig. 1). The number of labelled cells at 4-5 hours post-injection, counted both within and closely surrounding the axons, appeared higher in the medio-apical portion of regenerating nerves $(11.67\pm4.04\%; N=8)$. This value decreased to almost 0% (N=3) in the proximal, thicker nerves at 3-6 mm from the tail tip or close to the original tail stump (Fig. 2).

The cytoplasm of some enwrapping cells was oriented along or perpendicular with respect to the growing axons, and appeared to contact the naked axons (Figs. 1, 4-6). Other cells were actually wrapping one or more unmyelinated axons, as typical for Schwann cells (Figs. 5, 6).

In the apical nerves, sheath cells surrounded naked or unmyelinated axons, but at this stage it was not possible to distinguish Schwann from endoneurial cells. A basal lamina was not seen around the apical axons, but became evident around axons of the proximal nerve bundles, 21 days or later from tail amputation (Fig. 7).

Endoneurial cells, derived form the connective of the regenerating tail, were later identified in wider and more proximal nerve bundles, at 3-4 weeks of tail regeneration. Endoneurial cells featured long cytoplasmic arms which surrounded bundles of axons (Figs. 2, 7). Endoneurial cells were sometimes labelled, both after 4 hours till 12-20 days post-injection (Figs. 7, 8).

While in the apical nerves the %L decreased at 6 day post-injection ($5.50\pm1.23\%$; N=4), this value increased in the medio-proximal portion of nerves at 12 ($16.62\pm6.54\%$; N=9) and 20 days post-injection ($8.44\pm2.82\%$; N=4; Fig. 8).

In our experimental condition, the process of myelination of regenerating nerves began at 3-4 weeks of tail regeneration.

At about 46 days of tail regeneration (25 days after injection in regenerating tail 21 days old), numerous nerves entering the regenerated tail, appeared myelinated to different degrees.

Discussion

The present observations suggest that the sheath cells of the regenerating nerves in the two species of lizard here examined, have a double origin, from preexisting Schwann cells of the original nerves and from the recruitment of Schwann and endoneurial cells from the regenerating blastema.

The proliferative ability of Schwann cells is well known (Asbury, 1967; Cox, 1969; Bunge, 1983; Pannese, 1974; Pannese et al., 1987). However, the nerve recruitment of Schwann and endoneurial cells from the blastema, is a new observation in lizard, while it has already been reported in the regenerating tail of the newt (Arsanto et al., 1992). Experimental studies on bird embryos have also shown that endoneurial and perineurial cells were derived from the mesenchyma of the limb bud, while Schwann cells derived from the neural crest (Haninec, 1988; Halata et al., 1990).

The increase of labelled Schwann cells in medioproximal nerves indicates that in these regions, proliferation is continuous but not fast enough to dilute the label. In fact, in other rapidly proliferating tissues of the regenerating tail, no accumulation of ³H-thymidine labelled cells has been observed 6-20 days after injection (Alibardi, 1994c,d). Conversely, regenerating apical nerves grow fast, therefore they do not accumulate labelled cells after 6-20 days post-injection. As a consequence, the %L in apical nerves decreased, from the initial 11.67% to 5.5% at 6 days post-injection, and to almost 0% at 20 post-injection days.

The presence of labelled cells in medio-proximal nerves at 4-6-20 days post-injection, suggests that migrating and proliferating sheath cells accumulate within these nerves. This indicates that nerves located in medio-proximal regions of a regenerating tail do not grow very fast. The accumulation of labelled sheath cells is associated to their enlargement and fasciculation by



Fig. 1. Anolis carolinensis, growing nerve (n) near the apical tip of a regenerating tail. Labelled cells are visible both inside the nerve and outside, among the blastematic cells (b) at 4 hours postinjection. x 540

Fig. 2. Anolis carolinensis, cross section of a forming nerve bundle (n) in medial level of a regenerating tail (about 5 mm from the tail tip). Unmyelinated and myelinated (arrows) axons are seen at about 25 days of tail regeneration. A labelled Schwann cell is visible at 4 hours post-injection (arrowhead). x 830

Fig. 3. Lampropholis delicata, labelled satellite cell (arrow) at 6 day post-injection in the first proximal ganglion innervating a regenerating tail. The arrowhead points to a myelinated axon. N: ganglion neurons. x 1,200

Fig. 4. Anolis carolinensis, unlabelled and labelled mesenchymatous cells (arrows) close to a nerve (n) of the blastema. x 4,400 endoneurial cells.

Also during nerve regeneration in the newt, Schwann or endoneurial cells derive from the blastema or from mesenchymal cells located among the regenerating muscles (Arsanto et al., 1992). The latter cells precede the growing axons, and are thought to derive from the migration of Schwann cells into the forming blastema during the first days post-trauma, when there are no regenerating axons to colonize the blastema. The migration of Schwann cells into the regenerating blastema of *Ambystoma* has been well documented, as well as their potential metaplastic transformation in other cell types (Wallace, 1972).

Other studies in rat and mouse, have also indicated



Fig. 5. Anolis carolinensis, labelled Schwann cell enwrapping two unmyelinated axons (A). x 13,600

Fig. 6. Anolis carolinensis, Schwann cell at a late stage of cell division. The arrowhead points to the «division midbody» with its cleavage furrow (see inset at x 11,500). A: axons. m: labelled mesenchymal cell surrounding the axons. x 5,100

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that Schwann or ensheathing cells can multiply and migrate for long distances, preceding the growing axons (Díaz-Flores et al., 1995; Doucette, 1995).

As regards lizard, various studies have shown that the blastema is derived from the migration of mesenchymal cells from various injured connective tissues, including the connectival sheaths of truncated nerves (Simpson, 1965; Cox, 1969; Alibardi and Sala, 1983). Despite the more or less uniform appearance of lizard blastematic cells (Alibardi and Sala, 1988), most of the past studies have indicated that cells in the blastema redifferentiate according to their tissue of

Fig. 7. Anolis carolinensis, labelled endomysial cell at 4 hours post-injection in a medial nerve. The rim of cytoplasm (r) delimits the space occupied by amyelinic axons (A). S: cytoplasm of Schwann cells. In the inset at x 800 two labelled endomysial cells (arrows) surround the nerve fascicles (n) where unmyelinated and myelinated (arrowheads) axons are visible. x 24,900

Fig. 8. Lampropholis delicata, electron microscopic view of myelinated (arrowheads) nerve (n in the inset at x 760), located at about 6 mm from the tip of a regenerating tail. Labelled Schwann cells are seen at 12 days postinjection. x 8,300

origin (Bellairs and Bryant, 1985).

The present study also shows that a few labelled satellite cells in the spinal ganglia proximal to the regenerating tail, are visible at 4-12 days post-injection. This phenomenon is to correlate with the volumetric increase of the 2-3 ganglia in the original tail proximal to the regenerating tail, mostly due to the cell hypertrophy of spinal neurons (Terni, 1920; Pannese, 1963). The number of axo-somatic synapses in these neurons is also enhanced, and the cell hypertrophy is correlated to the greater increase of the peripheral innervation of these neurons, which extend their axons into the regenerating tail (Duffy et al., 1992). In fact, no peripheral nerves in the regenerated tail are derived from the regenerating spinal cord, which is encased inside a cartilaginous tube (Hughes and New, 1959; Simpson, 1965; Cox, 1969; Alibardi and Sala, 1983; Bellairs and Bryant, 1985). The innervation of the tissues of the regenerated tail is, instead, sustained by nerves coming from the native spinal cord and ganglia, closer to the regenerating tail (Terni, 1920; Zannone, 1953).

It is likely that satellite cells follow the increasing area and volume of the hypertrophic neurons by cell division, since the enhancement of the neural cell diameter is a stimulus to the multiplication of satellite cells (Pannese, 1974).

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References

- Alibardi L. (1994a). Fine autoradiographical study on scale morphogenesis in the regenerating tail of lizards. Histol. Histopathol. 9, 119-134.
- Alibardi L. (1994b). Modifications of the dermis during scale regeneration in the lizard tail. Histol. Histopathol. 9, 733-745.
- Alibardi L. (1994c). ³H-thymidine labelled cerebrospinal fluid contacting cells in the regenerating spinal cord of the lizard *Lampropholis*. Ann. Anat. 176, 347-356.
- Alibardi L. (1994d). Production of immature erythrocytes in lizard during tail regeneration. Anim. Biol. 3, 139-147.
- Alibardi L. and Miolo V. (1990). Fine observation on nerves colonizing the regenerating tail of the lizard *Podarcis sicula*. Histol. Histopathol. 5, 387-396.
- Alibardi L. and Sala M. (1983). Distribuzione di sostanze d'importanza morfogenetica in tessuti rigeneranti di *Lacerta sicula, Triturus alpestris* e *Rana dalmantina*. Atti Mem. Acc. Patav. Sci. Lett. e Arti. 95, 100-151.
- Alibardi L. and Sala M. (1988). Fine structure of the blastema in the regenerating tail of the lizard *Podarcis sicula*. Boll. Zool. 55, 307-313.
- Arsanto J.P., Komorowsky T.E., Dupin F., Caubit X., Diano M., Geraudie J., Carlson B. and Thouveny Y. (1992). Formation of the peripheral nervous system during tail regeneration in urodele amphibians: ultrastructural and immunocytochemical studies of the

origin of the cells. J. Exp. Zool. 262, 273-292.

- Asbury A.K. (1967). Schwann cell proliferation in developing mouse sciatic nerve. J. Cell Biol. 34, 735-743.
- Bellairs A. d'A. and Bryant S.V. (1985). Autotomy and regeneration in reptiles. In: Biology of the reptilia. Vol. 15 (Development B). Gans C. and Billet F. (eds). John Wiley & Sons Inc. New York. pp 301-410.
- Bunge R.P. (1983). Aspects of Schwann cell and fibroblast function relating to central nervous system regeneration. In: Spinal cord reconstruction. Kao C.C., Bunge R.P. and Reier P.J. (eds). Raven Press. New York. pp 261-270.
- Cox P.G. (1969). Some aspects of tail regeneration in the lizard Anolis carolinensis I). A description based on histology and autoradiography. J. Exp. Zool. 171, 127-150.
- Díaz-Flores L., Gutiérrez R., Varela H., Evora P., Valladares F., Rodríguez M., Rancel N. and Alvarez-Argüelles H. (1995). Contribution of the proximal and distal nerve stumps to peripheral nerve regeneration in silicone chambers. Histol. Histopathol. 10, 937-946.
- Doucette R. (1995). Olfactory ensheathing cells: potential for glial cell transplantation into areas of CNS injury. Histol. Histopathol. 10, 503-507.
- Duffy M.T., Liebich D.R., Garner L.K., Hawrych A., Simpson S.B. Jr. and Davis B.M. (1992). Axonal sprouting and frank regeneration in the lizard tail spinal cord: correlation between changes in synaptic circuitry and axonal growth. J. Comp. Neurol. 316, 363-374.
- Halata Z., Grim M. and Christs B. (1990). Origin of spinal cord meninges, sheaths of peripheral nerves, and cutaneous receptors including Merkel cells. Anat. Embryol. 182, 529-537.
- Haninec P. (1988). Study of the origin of connective tissue sheaths of peripheral nerves in the limb of avian embryos. Anat. Embryol. 178, 553-557.
- Hughes A. and New D. (1959). Tail regeneration in the geckonid lizard Sphaerodactylus. J. Embryol. Exp. Morphol. 7, 281-302.
- Pannese E. (1963). Investigations on the ultrastructural changes of the spinal ganglion neurons in the course of axon regeneration and cell hypertrophy. I) Changes during axon regeneration. Z. Zellforsch. 60, 711-740.
- Pannese E. (1974). The histogenesis of the spinal ganglia. Adv. Anat. Embryol. Cell Biol. 47, 7-92.
- Pannese E., Ledda M., Arcidiacono G., Frattola D., Rigamonti L. and Procacci P. (1987). Mitotic Schwann cells in normal mature spinal roots. La Cellule 74, 173-178.
- Simpson S.B.Jr. (1965). Regeneration of the lizard's tail. In: Regeneration in animals and related problems. Kiortis V. and Trampbush H.A.L. (eds). North Holland. Amsterdam. pp 431-443.
- Terni T. (1920). Sulla correlazione fra ampiezza del territorio di innervazione e grandezza delle cellule gangliari. 2. Ricerche sui gangli spinali che innervano la coda rigenerata nei sauri (Gongylus ocellatus). Arch. Ital. Anat. Embryol. 17, 507-543.
- Terni T. (1922). La rigenerazione del simpatico nella coda rigenerante dei sauri. Mon. Zool. Ital. 33, 58-62.
- Wallace H. (1972). The components of regrowing nerves which support the regeneration of irradiated salamander limbs. J. Embryol. Exp. Morfol. 28, 419-435.
- Zannone L. (1953). Fenomeni rigenerativi nervosi in rapporto con la rigenerazione della coda nel geko (*Tarentola mauritanica*). Ric. Morphol. 22, 183-200.

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