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Retinoic acid-dependent attraction of adult spinal cord axons towards regenerating newt limb blastemas in vitro

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Abstract

Adult urodele amphibians possess the unique ability to regenerate amputated limbs and to re-innervate these regenerating structures; however, the factors involved in mediating this re-innervation are largely unknown. Here, we investigated the role of retinoic acid (RA) and one of its receptors, RARβ, in the reciprocal neurotropic interactions between regenerating limb blastemas and spinal cord explants from the adult newt *Notophthalmus viridescens*. First, we showed that retinoic acid induced directed axonal outgrowth from cultured spinal cord tissue. This RA-induced outgrowth was significantly reduced when spinal cord explants were pre-treated with either the synthetic RAR pan antagonist, LE540, or the specific RARβ antagonist, LE135. The role of RARβ was also investigated using co-cultured regenerating limb blastemas and spinal cord explants. Blastemas induced significantly more axonal outgrowth from the near side of co-cultured explants, than from the far side (when cultured less than 1 mm apart). This blastema-induced directed outgrowth from co-cultured spinal cord explants was also abolished in the presence of the RARβ antagonist, LE135. These data strongly suggest that endogenous retinoic acid is one of the tropic factors produced by the blastema and that it may be capable of guiding re-innervating axons to their targets. Moreover, this interaction is likely mediated by the retinoic acid β nuclear receptor.

Keywords: Regeneration; Neurotrophic factors; Newt; Retinoic acid; Blastema; Axonal outgrowth; Regeneration; RARβ

Introduction

Adult urodele amphibians possess a unique capability: the ability to regenerate a number of lost structures such as limbs and spinal cord (Nye et al., 2003). In order to produce a functional limb after amputation, newly formed structures must be re-innervated by appropriate nerves (Singer, 1952). Therefore, a regenerating limb must produce an environment conducive to axonal growth, which would provide appropriate cues to the axons, allowing for accurate target innervation (Wilson et al., 1989).

It has been suggested that during limb regeneration in urodeles, correct re-innervation requires reciprocal signaling between re-innervating nerves and the undifferentiated mass of mesenchymal cells known as the regeneration blastema (Bauduin et al., 2000; Pollack et al., 1981). Re-innervating nerves from the spinal cord may provide mitogenic factor(s), such as substance P, transferrin, glial growth factor, and/or fibroblast growth factor to the blastema cells, in order to stimulate and maintain their proliferation (Stocum, 2004). In turn, to guide re-innervating nerves to their appropriate targets, the blastema may produce diffusible neurotropic molecule(s) (Bauduin et al., 2000; Tonge and LeClere, 2000). Many neurotrophins have been ruled out as the chemoattractants in the reciprocal interaction between regenerating limb blastemas and spinal cord neurons (Tonge and LeClere, 2000). However, the role of small diffusible non-protein factors, such as the Vitamin A metabolite, retinoic acid (RA), has not yet been conclusively determined.

There is currently strong evidence to support a role for RA in growth and differentiation of many embryonic cell

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types (Maden and Hind, 2003). In particular, the chick embryonic spinal cord is a rich source of RA (Maden et al., 1998) and RA-deficient quail embryos show little or no outgrowth from the spinal cord to the periphery (Maden et al., 1996). RA can direct neurite outgrowth from embryonic chick dorsal root ganglia neurons in vitro (Maden et al., 1998), and induces neurite outgrowth from larval salamander (Hunter et al., 1991) and embryonic mouse spinal cord (Corcoran et al., 2002) cultures. RA-induced neurite outgrowth is mediated by members of the RAR family of nuclear receptors (Corcoran et al., 2000), particularly through the upregulation of a specific receptor subtype, RARβ2 (Corcoran et al., 2002).

However, in the adult mouse, cultured spinal cord neurons do not extend axons even in the presence of RA, nor do they upregulate RARβ2, presumably due to transcriptional inactivation of this gene in the adult (Corcoran et al., 2002). Urodele amphibians, on the other hand, differ from these species described above, in that they can regenerate CNS tissues in the adult. Though RA has been shown to play a role in neurite outgrowth from larval axolotl spinal cord in vitro (Hunter et al., 1991), its precise role in regeneration from the adult spinal cord is unclear. We recently provided evidence that the neurotropic activity of cultured limb blastemas on spinal cord neurons from the adult newt may involve endogenous RA (Prince and Carlone, 2003). In this previous study, blastemas were pre-treated with citral, an inhibitor of retinaldehyde dehydrogenase (a key enzyme in the synthesis of endogenous RA; Duester, 2000), and then co-cultured with spinal cord explants. We showed that the neurotropic activity of the citral-treated blastemas was reduced by more than 40% (Prince and Carlone, 2003). This previous study strongly suggested that RA was an important factor involved in regeneration of adult spinal cord explants, though the possibility that RA was exerting indirect effects was not ruled out. In the present study, our main aim was to investigate the direct role of RA in the neurotropic and chemotactic interaction between regenerating limb blastemas and spinal cord explants from the adult newt, *Notophthalmus viridescens*. In particular, with the use of selective RA receptor antagonists, we aimed to determine whether blastema-derived retinoic acid induced directed neurite outgrowth from spinal cord explants by acting through the RARβ receptor.

Materials and methods

Limb amputation and blastema removal

Adult red-spotted newts, *N. viridescens*, were supplied by Boreal Labs (St. Catharines, ON). All protocols were approved by the Brock University Animal Care and Use Committee. Newts were anesthetized by placing them in an aqueous solution of 3-amino benzoic acid ethyl ester (0.1% w/v) (Sigma). The skin was sterilized by brief immersion in chloramine T (0.1% w/v) (Sigma) followed by a brief rinse in sterile amphibian Ringer's solution. All equipment used during spinal cord removal was sterilized in 70% ethanol and flamed. Limb amputations were made just proximal to the wrist of both forearms. Early blastemas were removed 8 days after the original amputation for primary blastemas. In order to test whether the quality or quantity of neurotropic factors differ after reamputation of the blastema at the stump–blastema interface, secondary blastemas were removed 6 days after the amputation of primary blastemas. Secondary blastemas reach equivalent morphological stages more quickly than primary blastemas (Wallace, 1981). Blastemas were immediately immersed in amphibian Ringer's solution containing antibiotic and antimycotic (GIBCO). They were then briefly placed in chloramine T solution (1.0% w/v) followed by a rinse in sterile amphibian Ringer's solution (with antibiotic and antimycotic), and then cultured.

Spinal cord removal and culture

After anesthesia and sterilization, newts were decapitated and the spinal column was carefully dissected along the full length of the body. The spinal column was sterilized by brief immersion in chloramine T (0.1% w/v), followed by a rinse in sterile amphibian Ringer's solution (with antibiotic and antimycotic). Vertebrae were pulled apart with fine forceps to remove the spinal cord from the spinal column. The isolated spinal cord was placed in amphibian Ringer's solution containing antibiotic and antimycotic for 10 min. While in the Ringer's solution, the meninges were removed with fine forceps and the spinal cord was cut with a scalpel into transverse sections approximately 0.5 mm thick. The spinal cord sections were then submerged in a chloramine T solution (0.1% w/v) for 30 s to ensure sterility, rinsed in sterile amphibian Ringer's solution (with antibiotic and antimycotic), and then rinsed in L-15 defined medium. Spinal cord sections were then cultured immediately, as explained below.

Culture conditions

Spinal cord explants were cultured on poly-L-lysine-coated (Sigma) glass coverslips (Belco) in 35-mm culture dishes (Fisher). A maximum of three spinal cord sections were cultured in each dish. To facilitate attachment of the spinal cord to the dish, the explants were cultured for the first 10 min in a minimal volume of medium. Once the spinal cord became attached, the volume of the medium was adjusted to 3 ml, which was sufficient to cover the explant. Explants were cultured in 70% Leibovitz L-15 medium (GIBCO). The defined medium was supplemented with 1% glucose, 300 ng/ml glutamine, 125 U/ml penicillin, 125 μg/ml streptomycin, 0.14 U/ml insulin (all GIBCO), 6.3 ng/ml progesterone (Sigma), and 5 μg/ml

transferrin (Sigma). The pH of the culture medium was adjusted to 7.5–7.6.

Due to the light-sensitivity of RA, all-*trans*-RA (Sigma) stocks were prepared fresh in absolute ethanol in the dark. The treatment of spinal cord explants with RA and/or various chemicals was performed immediately following attachment. Control cultures received the appropriate volume of EtOH vehicle. The cultures were maintained at 25°C ± 1°C in a humidified atmosphere in the dark.

Synthetic RA receptor antagonists LE135 and LE540 were kindly donated by S. Kagechika (U. of Tokyo). Both antagonists are dibenzodiazapine derivatives. LE135 can bind selectively to RARα and RARβ with higher affinity for RARβ (Eyrolles et al., 1994; Kagechika, 2002). LE135 inhibits the retinoic acid-induced differentiation of human promyelocytic leukemia cells (Eyrolles et al., 1994) at concentrations similar to those utilized in this study, and disrupts normal patterning during limb regeneration in the axolotl (Del Rincon and Scadding, 2002). Antagonists were dissolved in DMSO and diluted to the final concentration (2×10^{-6} M) in defined medium. Stock concentrations (2 mM) were stored at −20°C. Antagonists were added to spinal cord explants at least 1 h before the addition of either the blastemas or the RA. In the absence of RA and/or antagonists, vehicle (EtOH and DMSO, respectively) concentrations were adjusted to equal those containing the appropriate agents.

Chemotaxis: RA beads or blastemas co-cultured with newt spinal cord explants

AG 1-X2 beads (BioRad) were washed several times in 1 × PBS buffer, and soaked in RA (4 mg/ml) dissolved in absolute ethanol. Vehicle control beads were soaked in absolute ethanol. All beads were soaked for 72 h in the dark and stored at −20°C. Beads were washed three times in culture medium, and plated in separate dishes for each experiment, no further than 1 cm from the spinal cord explants. Blastemas (primary or secondary) were plated approximately 1 mm from newly attached spinal cord explants. All blastema–spinal cord co-cultures contained DMSO, as it was the vehicle solution for the antagonist used in the study.

Analysis of axon outgrowth

All cultures were examined for axonal outgrowth 72 h following spinal cord culture. Cultures were viewed with a Zeiss Axiovert 200 inverted microscope, using a digital camera and Northern Eclipse (Empix Imaging, ON) imaging software. All results were expressed as mean ± the standard error of the mean. In RA and antagonist cultures, data were analyzed using a one-way analysis of variance (ANOVA) and Dunn's Post Hoc test, and considered significant when $P < 0.05$. For blastema and/or RA-bead co-cultures, the difference in number of axons between each side of the explant was calculated in each condition and the values compared using a 1-way ANOVA, and Dunn's Post Hoc test, and considered significant when $P < 0.05$.

Results

RA induced directed neurite outgrowth from adult newt spinal cord explants in vitro

In order to determine whether RA induces outgrowth of neurites from the adult newt spinal cord, explants were cultured in either 10^{-7} M RA, defined medium alone or vehicle solutions (no treatment controls) and examined after 72 h in culture. Explants in control conditions extended few axons (defined medium: 23 ± 3.5 neurites, $n = 21$ explants, Fig. 1A; EtOH: 16.8 ± 2.3 neurites, $n = 16$ explants, Fig. 1B), but the number of axons was not significantly different between the two control conditions. In contrast, the RA-treated explants extended significantly more axons than controls (53.4 ± 8.2 neurites, $n = 13$ explants, $P < 0.01$, Fig. 1C). These results support our previous findings that RA induces neuronal outgrowth from adult newt spinal cord explants in vitro (Prince and Carlone, 2003).

In order to determine if RA acts as a chemoattractant for regenerating nerves, we cultured spinal cord explants with an exogenous source of RA, and monitored the explants for directed axonal outgrowth 72 h later. AG-1X2 beads, soaked in either RA or absolute ethanol, were plated to one side of the cultured spinal cord explants. The beads produce a continual source of either RA or vehicle (Tickle

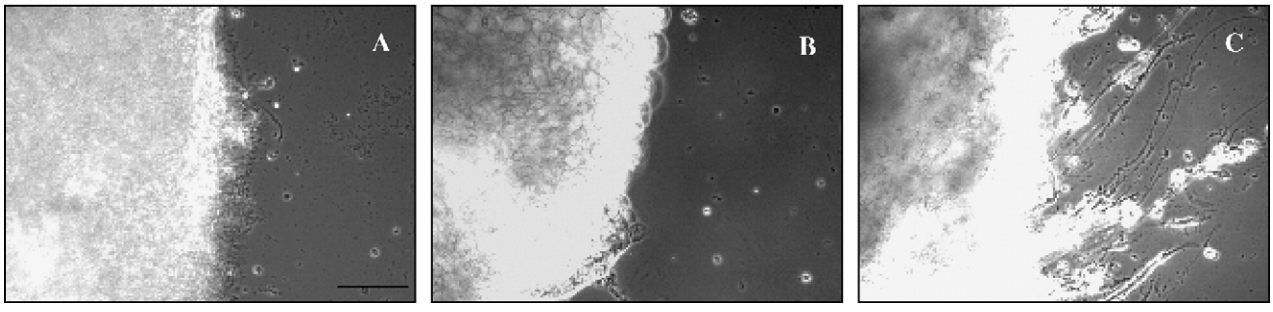

Fig. 1. RA induced axonal outgrowth in adult newt spinal cord explants. Photos taken 72 h after treatment with (A) defined culture medium, (B) EtOH, and (C) 10^{-7} M RA. Scale bar = 50 μm.

et al., 1982). We showed a significant increase in number of neurites on the side of the explant closest to the RA bead, compared to the side furthest from the bead ($P < 0.01$, $n = 23$ explants; Fig. 2). In contrast, there was no significant increase in number of neurites on the side of the explant closest to the control bead, compared to the side furthest from the control bead ($n = 27$ explants; Fig. 2). These results strongly suggest that a concentration gradient of RA affects the direction of neuronal outgrowth from the adult newt spinal cord explants.

Limb blastema-induced directed outgrowth from spinal cord explants

Our previous study (Prince and Carlone, 2003) suggested that retinoic acid produced by the blastema induces neurite outgrowth from spinal cord explants. We next wished to determine whether the regeneration blastema could also induce directed outgrowth (chemotaxis) from the cultured explants, and thus mimic the effects of RA shown above. For this purpose, we investigated the neurite-inducing effects of both primary and secondary blastemas. Primary blastemas were obtained from an original amputation whereas secondary blastemas arise after removal of the original limb blastema. There was significantly more outgrowth ($P < 0.01$) when spinal cord explants were co-cultured with primary blastemas (85 ± 7 neurites, $n = 27$ explants) or secondary blastemas (40 ± 7, $n = 29$ explants), than explants cultured in the absence of blastemas (23 ± 3.5 neurites, $n = 21$ explants). However, we also showed that primary blastemas induced significantly more outgrowth than secondary blastemas ($P < 0.01$). All culture conditions with blastemas contained DMSO (<0.1% w/v) as vehicle unless otherwise stated.

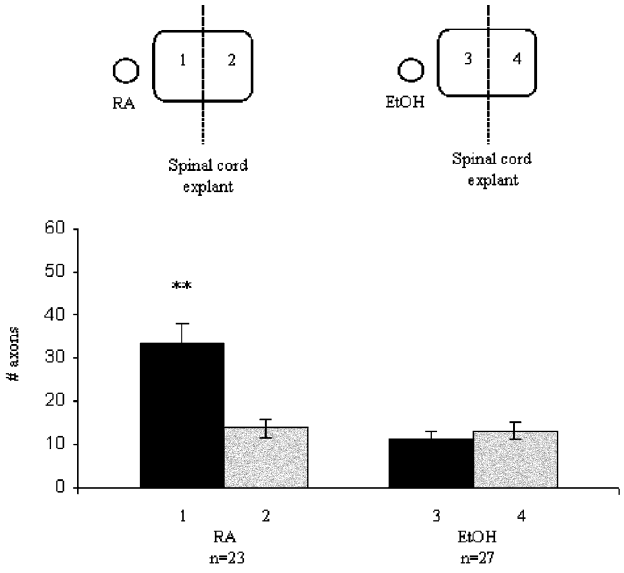

Fig. 2. RA-soaked beads induced directed axonal outgrowth. Above: diagrammatic representation of spinal cord and bead locations (RA 1, 2 or EtOH 3, 4). Below: graph depicting increase in axonal outgrowth from side of adult spinal cord explant closest to RA-soaked bead. **There was a significant increase in number of axons on the side of the explant exposed to the RA bead ($P < 0.01$) versus all other sides.

By comparing the neurite outgrowth from the explant side closest to the blastema, to the explant side farthest from the blastema, we were next able to determine whether either the primary or secondary blastema was capable of inducing directed outgrowth. Indeed, we found that directed outgrowth (chemotaxis) was observed when either the primary ($n = 14$ explants) or secondary blastemas ($n = 13$ explants) were positioned less than 1 mm from the explants ($P < 0.01$; Figs. 3A, B; see Table 1 for near side and far side data). To determine if the chemotactic response was distance dependent, the blastemas and spinal cord explants were next co-cultured up to 1 cm apart. At this distance, the blastema continued to induce outgrowth, but this outgrowth was not directed as it was not limited to one side of the explant (see Table 1). Thus, at distances greater than 1 mm, the chemotactic response of spinal cords to both primary and secondary blastemas was significantly diminished ($P < 0.01$; Figs. 3C, D).

These data strongly suggest that the blastema (both primary and secondary) can induce directed outgrowth that is distance dependent. This may be due to a concentration-dependent phenomenon of a diffusible factor, such as RA, capable of directing outgrowth. Fig. 4 demonstrates the ability of both RA-soaked beads and secondary blastemas, when cultured less than 1 mm from the explant, to direct neurite outgrowth.

RA-induced outgrowth is mediated by a member of the RARβ receptor family

RA has been shown to induce axon outgrowth via its nuclear receptor, RARβ (Corcoran and Maden, 1999). In order to determine whether RA-induced neuronal outgrowth is mediated by RARβ, spinal cord explants were cultured in the presence of RA and either LE540 (RAR α, β or γ) or LE135 (RARβ receptor antagonist; Fig. 5). In the presence of 10^{-7} M RA (and antagonist vehicle), spinal cords extended an average of 47 ± 4 axons ($n = 29$ explants; Fig. 5). This outgrowth was significantly decreased in the presence of both LE135 (13 ± 3 axons; $n = 28$ explants; $P < 0.01$; Fig. 5) and LE540 (27 ± 5 axons; $n = 29$ explants; $P < 0.05$; Fig. 5). The antagonists had no significant effect in the absence of RA (Fig. 5). There was also no significant difference in the number of axons found in the control medium containing only vehicle solutions (Fig. 5). These results strongly suggest that the RA-induced outgrowth from adult newt spinal cord explants was mediated by a member of the RARβ class of nuclear receptors.

Limb blastema-induced neuronal outgrowth in spinal cord explants is also mediated by RARβ

Here, we aimed to determine whether the directed outgrowth induced by either primary or secondary blaste-

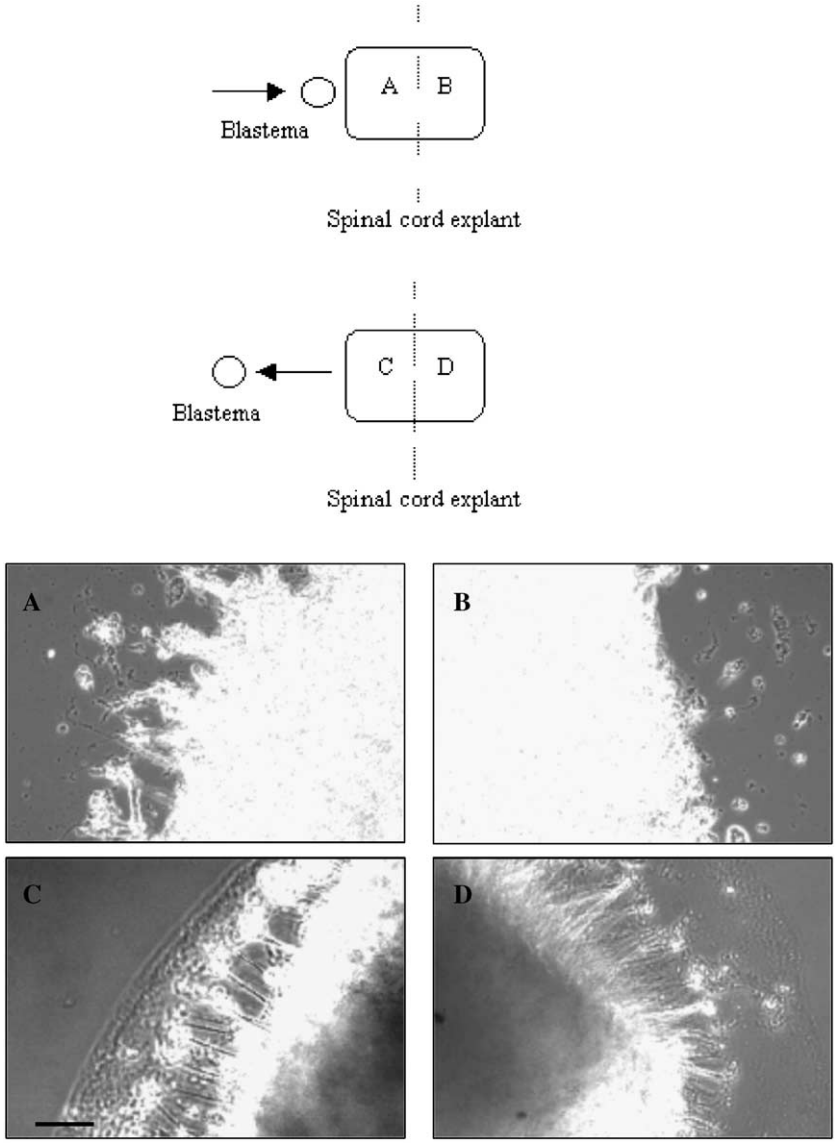

Fig. 3. Primary blastema and spinal cord co-cultures induced directed axonal outgrowth at distances less than 1 mm. Above: diagrammatic representation of spinal cord and blastema locations (less than 1 mm: A, B, or more than 1 mm: C, D). Below: representative photos of spinal cord explants taken 72 h after co-cultured primary blastema is (A) less than 1 mm to the left of an explant, where (B) shows the right side of the same explant, (C) more than 1 mm to the left of a different explant, where (D) shows the right side of the same explant. Scale bar = 50 μm.

mas was affected by the RARβ receptor antagonist. We found that the total number of axons extending from spinal cord explants co-cultured with a primary blastema was reduced by 61% in the presence of LE135 (33 $\pm$ 5 neurites, n = 33 explants, $P < 0.01$; Fig. 6). In secondary blastema co-cultures, LE135 reduced the total number of blastema-induced neurites by 67.5% to an average of 13 $\pm$ 3 (n = 27 explants, $P < 0.01$; Table 1). Furthermore, the outgrowth that was observed was no longer directed outgrowth. That is, in the presence of LE135, the number of neurites extending from the side of the explant closest to the blastema was no longer significantly different from that extending from the far side of the explant (see Table 1). These results strongly suggest that the neurite-inducing chemotactic effects of the regeneration blastema are mediated by RA acting through the RARβ receptor.

Discussion

Previous studies have shown that regenerating limb blastemas and limb buds from amphibians are capable of eliciting axon outgrowth from spinal cord and dorsal root ganglion explants (Bauduin et al., 2000; Prince and Carlone, 2003; Tonge and LeClere, 2000; Tonge et al., 2004). Despite attempts to elucidate their chemical nature, the identity of the nerve growth-inducing factors produced by limb buds or blastemas remains unclear.

Table 1
Primary blastema and spinal cord co-cultures induced significantly more total axonal outgrowth than secondary blastemas

Condition	# Axons	Near side # axons	Far side # axons	*n*
Explant alone	23 ± 4	n/a	n/a	21
Explant far blast 1° + DMSO	95 ± 9	46 ± 6	49 ± 6	13
Explant far blast 1° + LE135	39 ± 7	17 ± 4	22 ± 4	11
Explant close blast 1° + DMSO	75 ± 11	48 ± 7	27 ± 5	14
Explant close blast 1° + LE135	30 ± 6	17 ± 4	13 ± 3	22
Explant far blast 2° + DMSO	57 ± 11	30 ± 6	27 ± 6	16
Explant far blast 2° + LE135	17 ± 6	8 ± 4	9 ± 3	13
Explant close blast 2° + DMSO	18 ± 2	13 ± 2	5 ± 1	13
Explant close blast 2° + LE135	8 ± 2	4 ± 1	5 ± 1	14
Explant total[a] blast 1° + DMSO	85 ± 7	47 ± 4	38 ± 4	27
Explant total[a] blast 1° + LE135	33 ± 5	17 ± 3	16 ± 2	33
Explant total[a] blast 2° + DMSO	40 ± 7	22 ± 4	17 ± 4	29
Explant total[a] blast 2° + LE135	13 ± 3	6 ± 2	7 ± 2	27

[a] Total blastema data represent the average of the combined far and close co-culture data.

Our present results are consistent with our earlier work demonstrating a potential role for endogenous RA in regenerating limb blastema-induced axon outgrowth of adult spinal cord neurons (Prince and Carlone, 2003). We previously showed that when the synthesis of RA in the blastema mesenchyme was inhibited with citral (an inhibitor of retinaldehyde dehydrogenase), the axon outgrowth-promoting activity of the blastema diminished by approximately 40% (Prince and Carlone, 2003). This work strongly suggested that the blastema was producing RA, which could act to guide regenerating axons to their appropriate targets in the regenerating limb. Because RA is a small, lipophilic molecule, it seems likely that it could serve as a diffusible factor, capable of guiding regenerating neurons over significant distances. Indeed, both developing limb buds and regeneration blastemas contain retinoids, including RA (Scadding and Maden, 1994; Scott et al., 1994).

Maden et al. (1996), using an RA-deficient quail embryo model system, have demonstrated that RA is necessary for appropriate outgrowth of embryonic neurons in vivo. When quail embryos develop in the absence of endogenous RA, outgrowth from the neural tube is minimal, and if outgrowth is present, it is chaotic and misdirected.

In vitro, RA has been shown to elicit outgrowth and promote survival in axolotl and mouse embryo spinal cord cultures and also in DRG cultures from a variety of vertebrates (Corcoran et al., 2000; Hunter et al., 1991; Maden et al., 1998; Prince and Carlone, 2003). Experiments have also shown that RA can act as a chemoattractant molecule, capable of dictating the direction of outgrowth from chick embryo neural tube cells (Maden et al., 1998). All these data strongly support a role for RA in directed axonal outgrowth.

In contrast, Tonge and LeClere (2000) previously suggested that RA, either alone, or in combination with NGF, failed to induce axon outgrowth from axolotl DRG-peripheral nerve explants. It is possible that concentration, culture conditions, tissue-specific or species-specific differences could underlie the discrepancy in our results. For example, our explants were cultured in 10^{-7} M RA on low molecular weight poly-L-lysine. These conditions elicited a threefold increase in the number of axons compared to vehicle-treated controls. In contrast, the explants of Tonge and LeClere (2000) were cultured in lower concentrations of RA (10^{-8} to 10^{-9} M) and were maintained in matrigel. Hunter et al. (1991) previously showed that there was a threshold concentration of 10^{-8} to 10^{-9} M RA, below which stimulation of outgrowth from axolotl spinal cord neurons was significantly reduced. Furthermore, concen-

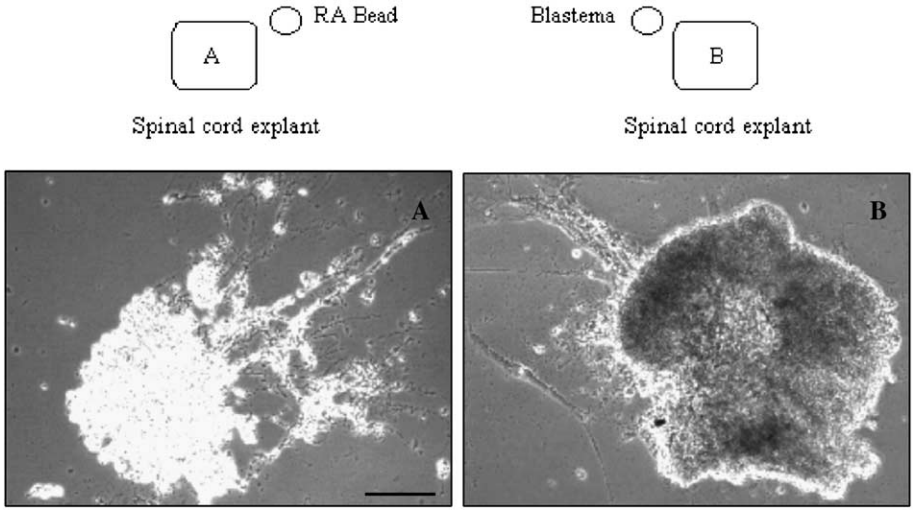

Fig. 4. Local application of RA or a closely cultured secondary blastema showed similar abilities to direct axonal outgrowth. Above: diagrammatic representation of (A) spinal cord and bead co-culture or (B) spinal cord and blastema co-culture. Below: (A) photo of spinal cord cultured to the lower left of an RA soaked bead (~300 μm). (B) Photo of another spinal cord explant cultured to the lower right of a secondary blastema (less than 1 mm). Explants were monitored 3 days after culture with beads or blastemas. Scale bar = 50 μm.

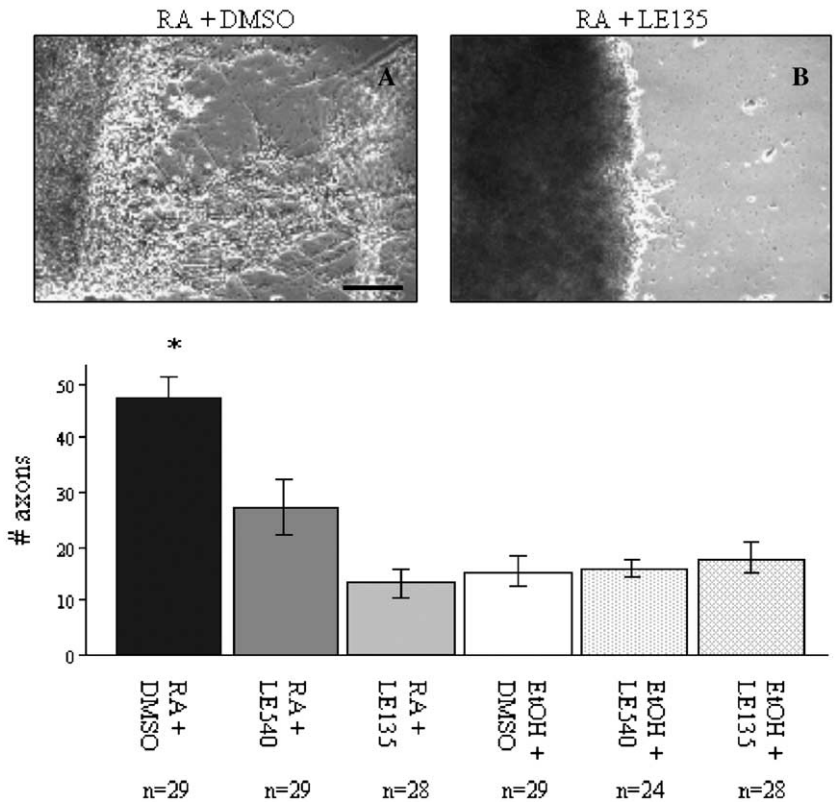

Fig. 5. RAR antagonists significantly decreased RA-induced axonal outgrowth. Above: representative photos of spinal cord explants treated with (A) RA and DMSO or (B) RA and LE135. Below: graph depicting significant reduction in RA-induced axons outgrowth in the presence of RAR antagonists. The number of axons was counted 72 h after treatment with RAR antagonists in the presence or absence of RA. *RA treatment elicited significantly more axons than antagonist solutions and controls ($P < 0.05$). Scale bar = 50 μm.

trations as high as 10^{-6} M have been effective (and are not toxic) in eliciting outgrowth from explants of the day 13.5 mouse embryo spinal cord (Corcoran et al., 2002). Scadding and Maden (1994) have also estimated the concentrations of endogenous RA in the posterior quadrant of urodele amphibian limb blastema mesenchyme to be greater than 10^{-7} M. This physiological concentration is the same as that used in the present study and in other studies showing effective growth and survival in RA.

In addition to our results on neural outgrowth, we have provided the first evidence that RA can act as a guidance factor to direct the growth of neurons from adult newt CNS tissue. We applied RA-soaked AG 1-X2 beads to cultures at distances up to 1 cm from the spinal cord explants. Axonal outgrowth was consistently directed towards the beads, and was predominantly seen in that half of the explant directly opposed to the bead. When beads were cultured closer, axons could be seen contacting the surface of the beads (data not shown). Previous studies demonstrating RA chemotaxis have employed dissociated embryonic neural tube cells cultured in a Dunn chamber (Maden et al., 1998). This apparatus provides a means of generating a stable concentration gradient of RA (or any other soluble molecule) in vitro (Zicha et al., 1991). Although we did not measure the production of stable gradients of RA in this study, AG 1-X2 beads are well known to provide a stable continual source of RA in vitro (Tamura et al., 1990).

As found by others, we also demonstrated that blastemas were capable of directing axon outgrowth (Bauduin et al., 2000; Tonge and LeClere, 2000; Tonge et al., 2004). In our case, directed outgrowth was only observed when blastemas were co-cultured within 1 mm of the spinal cord explant, a result consistent with that of Bauduin et al. (2000). At distances greater than 1 mm, axons were randomly distributed around the circumference of the spinal cord explant. The directed outgrowth was also seen when spinal cord explants were co-cultured with secondary blastemas (blastemas derived from a second amputation of the same limb). However, the chemotactic response and overall neurotrophic activity of secondary blastemas was significantly reduced compared to the effects of primary blastemas of equivalent size and morphology. Secondary blastemas regenerate much more rapidly than primary blastemas (Wallace, 1981). The molecular basis for this increased regeneration rate is unknown, but our results suggest that perhaps the reciprocal interaction between blastema mesenchyme and regrowing peripheral nerves is altered such that the secondary blastema produces less RA for axon outgrowth. In support of this idea, Scadding and Maden (1994) have shown that the endogenous concentrations of retinoids fluctuate both spatially and temporally in the blastema

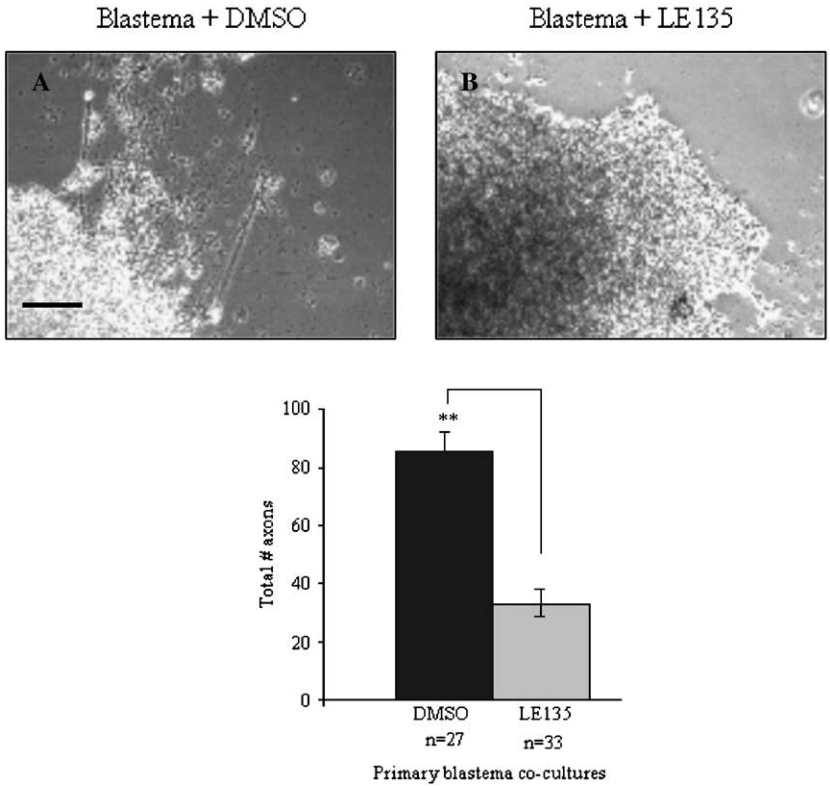

Fig. 6. RARβ antagonists significantly decreased total axonal outgrowth induced by primary blastemas. Above: (A, B) Representative photos of spinal cord explants and primary blastema co-cultures taken 72 h after treatment with (A) antagonist vehicle solution or (B) LE135. Blastema was cultured to the upper right of explant in pictured experiments (A and B). **Primary blastema co-cultures (DMSO control) showed significantly more axons when compared to primary blastemas and LE135 ($P < 0.01$). Scale bar = 50 μm.

during the course of regeneration, although no data on the levels of RA in secondary blastemas exist at this time.

It is possible that the blastema is producing multiple factors capable of guiding axons to their targets in the regenerating limb. Several factors, including the neurotrophins, are able to stimulate axon outgrowth in vitro. It is unlikely, however, that the limb blastema-derived activity can be wholly accounted for by the presence of neurotrophins such as NGF, BDNF, NT-3, NT-4, GDNF, HGF, and various FGFs found in these tissues. For example, experiments blocking neurotrophin receptors with k252a, or using trk-IgG chimeras to sequester the ligands, failed to inhibit the neurotropic activity produced by blastemas (Tonge and LeClere, 2000; Tonge et al., 2004). Bauduin et al. (2000) have also suggested that the neurotropic activity present in blastema-conditioned media resides in a low molecular weight (<15,000 Da), heat-stable protease-sensitive factor. Though RA fulfills the requirement of being a low molecular weight substance, it is, however, heat sensitive and protease insensitive.

In the present study, perhaps the most compelling argument in support of a role for endogenous, blastema-derived RA in the stimulation of neurite growth comes from our results with the retinoic acid receptor antagonist LE135. We showed that addition of the antagonist almost completely abolished the effects of 10^{-7} M RA on spinal cord axon outgrowth. More importantly, LE135 also completely abolished the stimulatory effects of co-cultured blastemas on both axon number and direction of growth. LE135 alone had neither a stimulatory nor an inhibitory effect on axonal outgrowth compared to both vehicle-treated and untreated control spinal cords.

These results with LE135 are consistent with the existence and expression of an RARβ isoform in the adult newt central nervous system. In the human, rat, and mouse, there are α, β, and γ subtypes of both the RARs and RXRs (which bind and mediate the effects of 9-cis retinoic acid), each with multiple isoforms. In the newt, both RARα and δ (which is equivalent to the mammalian RARγ) had previously been reported (Ragsdale and Brockes, 1991; Ragsdale et al., 1989, 1992; Maden and Hind, 2003). Until now, however, there was no consistent evidence supporting expression of an RARβ in the newt CNS (Maden and Hind, 2003). Del Rincon and Scadding (2002), however, have shown that LE135 inhibits limb regeneration in axolotls, suggesting a role for RARβ in the reestablishment of pattern during limb regeneration. We have recently cloned a newt RARβ homologue (Carter et al., 2005), and in preliminary studies, have demonstrated that its expression is maintained in the adult spinal cord and brain and is upregulated after amputation in both the tail and limb (unpublished). It appears from our results that this isoform mediates the

effects of RA on neuronal outgrowth in adult tissues as it does in the mammal during embryogenesis (Corcoran et al., 2002). In contrast to mammals, however, the expression of this newt receptor isoform is maintained in the adult spinal cord, and may contribute to the ability of adult newt CNS tissue to regenerate.

In conclusion, it is likely that the blastema produces multiple factors that act on regrowing peripheral nerves during the course of urodele limb regeneration, with specific factors perhaps affecting specific populations of neurons. The present data argue for a significant contribution of endogenous RA in this process and highlight the diverse nature of effects this small lipophilic molecule has in development and regeneration.

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