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Original Article

Disto-proximal regional determination and intercalary regeneration in planarians, revealed by retinoic acid induced disruption of regeneration

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ABSTRACT The mechanisms that define the body pattern during development and regeneration are the object of major concern in developmental biology. To understand the process and sequence of antero-posterior pattern formation of planarian body regions during regeneration, regenerating organisms were treated with exogenous retinoic acid, which affects development and regeneration in other systems, and the sequence of regional determination has been monitored by a specific molecular marker for the central region, which includes the pharynx. The sequence of gross regional specification have never been analysed in planarians using molecular regional markers or by direct disruption of the regeneration process. Exogenous retinoic acid administration on regenerating planarians disrupts anterior, but not posterior regeneration. The period of maximum sensitivity to exogenous retinoic acid is one day after amputation, during which time the determination of the head has been reported to occur. The data obtained allow us to suggest that gross regional specification during planarian regeneration is disto-proximal, from the regenerative blastema to the old stump, and thus takes place by intercalation of the central region between the anterior and posterior ones.

KEY WORDS: *platyhelminthes, planarian, regeneration, regionalisation, retinoic acid*

Introduction

The question of how a defined body pattern arises, including during regeneration, is one of the most significant problems in developmental biology. Pattern formation is extensively studied in various animal models, including invertebrates and vertebrates. Among them, freshwater planarians (Platyhelminthes, Turbellaria, Tricladida), display a remarkable ability to regenerate. Even a small fragment cut from the body can give rise to an intact animal. The process of planarian regeneration includes at least two distinct but closely linked events: formation of a regenerative blastema, and pattern formation (restoration). In adult planarians, totipotent stem cells, referred to as neoblasts, are the only cells that proliferate (Baguñà *et al.*, 1989; for a general review, see Baguñà, 1998). After amputation, neoblasts from the old stump proliferate extensively, and migrate to the wounded surface forming the regeneration blastema (Saló and Baguñà, 1984). Structures to be regenerated form from both the cells of the regeneration blastema and neoblasts within the old stump, through an epimorphic-morphallatic mechanism (Saló and Baguñà, 1984).

Re-specification of the pattern along the antero-posterior (AP) body axis is the main event in any regenerating system. One of the main points of debate is whether this process occurs sequentially or simultaneously along the AP (head-tail) body axis and, in the former case, whether it is proximo-distal or disto-proximal. Transplantation methods used to study the determination of missing anterior structures in caudal regenerates (Saló, 1984; for a general review see Baguñà *et al.*, 1994) have shown that head determination is a very early event, occurring at 6-24 hours of regeneration, whereas determination of the pharynx occurs slightly later, at 12-36 hours of regeneration. Both events occur within a narrow strip of tissue of less than 500 μm below the wound, when the blastema is just barely visible. The determination of these structures may be causally related and may occur in a disto-proximal manner (Baguñà *et al.*, 1994; for a general review see Baguñà, 1998), in agreement with Wolff's model (Wolff *et al.*, 1964). These events have never been analysed in planarians using molecular regional markers or by direct disruption of the regeneration process.

One of the experimental approaches followed to study AP patterning restoration and to analyse the sequence of regional determination during regeneration is the experimental modification

Abbreviations used in this paper: AP, antero-posterior; mAb, monoclonal antibody; RA, retinoic acid.

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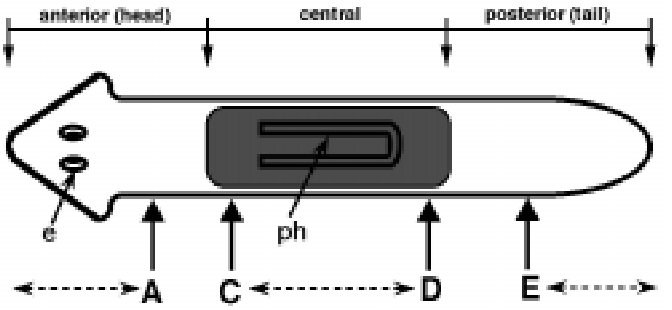

Fig. 1. Diagram of a freshwater planarian indicating AP molecular body regions and levels of body cut. (Top) *The three AP molecular body regions defined by TCEN49 localisation (in grey).* **(Bottom)** *Levels of body cut. Note that all regenerates have to regenerate two complete molecular body regions. Abbreviations: e, eye; ph, pharynx.*

of AP body regions by means of chemicals. Among the different chemicals described to have effects on developmental and regeneration processes, retionoic acid (RA) is probably the most efective. Most studies on RA effects have been performed on vertebrate development and regeneration. There are few data on invertebrates and, in fact, the possible specific effects of retinoic acid in invertebrates generate an intense debate. For example, RA treatment during development causes dose-dependent effects in molluscs (Créton *et al*., 1993), ranging from minor defects in eye size to developmental arrest as trocophora larva. In *Hydractinia echinata*, exogenous application of RA also leads to developmental defects (Müller, 1984). In both examples, the period of RA sensibility is very restricted: mid-blastula and gastrulation in molluscs and the first stages of metamorphosis in *Hydractinia*.

Our aim was to disrupt planarian regeneration by treating regenerating organisms with exogenous RA. To monitor planarian regional regeneration at a molecular level, we used a monoclonal antibody (mAb TCEN-49) that is specific for a small secreted protein (TCEN49) present only in the central region of the animals, including the pharynx (Bueno *et al*., 1996, 2001). On the basis of the presence/absence of TCEN49, three molecular body regions can be clearly distinguished along the AP body axis: anterior (including eyes and brain), central (including the pharynx) and posterior. Owing to its dynamics, the TCEN49 localisation during regeneration (Bueno *et al*., 1996) is an excellent molecular marker to monitor the regeneration of these gross molecular body regions. The data obtained show that exogenous RA affects anterior but not posterior regeneration in planarians; that the period of RA sensitivity is very restricted and coincides with the period of head determination; and suggests that the regional specification during planarian regeneration is disto-proximal and thus takes place by intercalation of the central region between the anterior and posterior ones.

Results

Daily treatments

To analyse the effects of exogenous RA administration on planarian regeneration, organisms cut at level A (heads), E (tails)

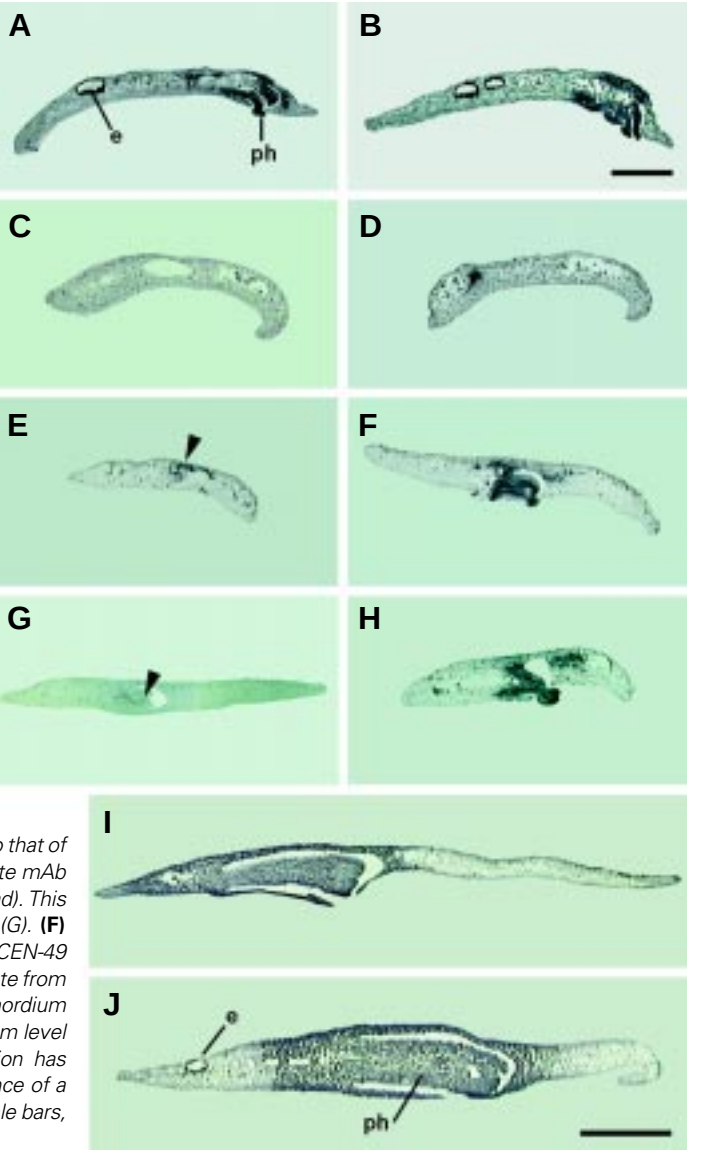

Fig. 2. Planarian sagittal sections of regenerates derived from the first set of experiments (daily treatments, 2 hours/day during 10 days) immunostained with mAb TCEN-49. *B,G and I are control organisms. Organisms were sacrificed after 10 days of regeneration. Dorsal is to the top, and anterior to the left.* **(A)** *Regenerate from level A. Note that the extent of regeneration and mAb TCEN-49 immunostaining is as in the control (B).* **(B)** *Control regenerate from level A.* **(C)** *Regenerate from level E. Note the total lack of a new pharynx and mAb TCEN-49 immunostaining.* **(D)** *Regenerate from level E. Note the initial mAb TCEN-49 immunostaining, and the total absence of a new pharynx. This regeneration stage corresponds to that of a 2-3 day-old control regenerate (not shown).* **(E)** *Regenerate from level E. Note mAb TCEN-49 immunostaining and the presence of a pharynx primordium (arrowhead). This regeneration stage corresponds to that of a 4-6 day-old control regenerate (G).* **(F)** *Regenerate from level E. Note that the extent of regeneration and mAb TCEN-49 immunostaining is as in a 10 day-old control (H).* **(G)** *5-6 day-old control regenerate from level E. Note mAb TCEN-49 immunostaining and the presence of a pharynx primordium (arrowhead).* **(H)** *10 day-old control regenerate from level E.* **(I)** *Regenerate from level C-D. Note the absence of the anterior region, and that the posterior region has regenerated normally.* **(J)** *Control regenerate from level C-D. Note the presence of a complete new anterior region, including the eye. Abbreviations as in Fig. 1. Scale bars, A-G, 0.5 mm; H and I, 1 mm.*

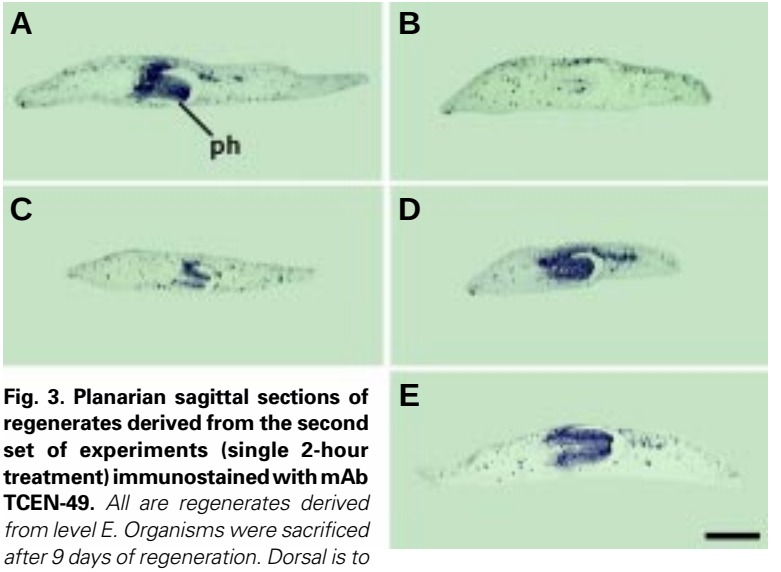

Fig. 3. Planarian sagittal sections of regenerates derived from the second set of experiments (single 2-hour treatment) immunostained with mAb TCEN-49. *All are regenerates derived from level E. Organisms were sacrificed after 9 days of regeneration. Dorsal is to the top, and anterior to the left.* **(A)** *Regenerate treated with exogenous RA at day 0, just after amputation.* **(B)** *Regenerate treated at day 1 after amputation. The pharynx primordium and the extent of mAb TCEN-49 immunostaining correspond to that of a 4 -5 day-old control regenerate (not shown, but see Fig. 3G for comparison).* **(C)** *Regenerate treated at day 2 after amputation. Note that the regeneration stage is slightly delayed compared with the control organism (E).* **(D)** *Regenerate treated 3 days after amputation.* **(E)** *Control regenerate. Abbreviations as in Fig. 1. Scale bar, 0.5 mm.*

and C-D (trunks) (see Fig. 1 for levels of amputation) were treated for 2 hours/day during 10 days, from day 0 (just after amputation) to day 9 of regeneration. Application of exogenous RA on intact adult planarians did not have any visible effect on morphology or on their molecular AP body regions, as revealed by mAb TCEN-49 immunostaining (not shown).

Organisms cut at level A (heads that may regenerate new central and posterior regions, including the pharynx) treated daily with exogenous RA were not different from controls, and the chronology of the process of regeneration was normal. Their molecular body regions, as revealed by mAb TCEN-49 immunostaining, were also normal (Fig. 2 A,B).

Organisms cut at level E (tails that may regenerate new central and anterior regions, including the pharynx, eyes and brain) treated daily with exogenous RA showed an arrest in regeneration. After 10 days of regeneration, regenerates could be classified in four categories: (I) regenerates that did not regenerate the pharynx, eyes or brain, and that did not generate new molecular central and anterior regions (as detected by mAb TCEN-49 immunostaining) (27.5%; Fig. 2C); (II) regenerates that started the process of molecular regionalisation but that regenerated neither the pharynx nor the eyes, which corresponded to 2-3 day-old control regenerates (not shown) (22.5%; Fig. 2D); (III) regenerates that started the process of molecular regionalisation and the regeneration of the pharynx, eyes and brain, but whose regeneration stage corresponded to a 4-6 day-old control regenerate (15.0%; Fig. 2 E,G); and (IV) regenerates that regenerated normally, as controls (35.0%; Figs 2 F,H). If regenerates were treated for more than 10 days, the most affected organisms (category I) died. On the other hand, if the treatment was cut off at any stage before the dying of the organisms, regenerates were mostly able to resume regeneration, with the corresponding delay. In these cases, organisms regenerated completely normal bodies.

The dynamics of cell accumulation in the regeneration blastema of RA treated organisms (not shown) was always parallel to reported morphological and molecular defects, i.e. marked disruption of regeneration led to smaller regeneration blastema. Organisms cut at levels C and D (trunks that may regenerate new anterior and posterior regions) treated daily with exogenous RA regenerated a new complete posterior region. However, they had the same defects in the regeneration of the anterior region (lack of eyes, brain and anterior molecular region) as described for level E regenerates, and in similar proportions (Figs. 2 I,J, and data not shown). Exogenous RA affects anterior but not posterior regeneration in planarians.

Single treatments

To analyse the period of RA sensitivity during anterior regeneration, organisms cut at level E (tails) were treated for 2 hours, once between day 0 (just after amputation) and 7 of regeneration, and sacrificed after 9 days of regeneration. Regenerates treated on day 0 and from day 3 onwards did not show morphological or molecular (in TCEN49 localisation) differences as compared with control organisms (Figs. 3 A,D and E) and regeneration proceeded normally.

However, regenerates treated with exogenous RA on day 2 or, especially, on day 1 of regeneration, showed a significant delay in regeneration. After 9 days of regeneration, the development of the pharynx and the expression of TCEN49 in the organisms treated on day 1 after amputation (Fig. 3B) were similar to those of control organisms after 4-5 days of regeneration (not shown, but see Fig. 2G for comparison). Organisms treated on day 2 of regeneration (Fig. 3C) showed a slight delay in regeneration: the development of the pharynx and the expression of TCEN49 were similar to those of control organisms after 7 days of regeneration (not shown). In these organisms, the process of regeneration took longer than in controls to be completed: 4-5 days longer for organisms treated on day 1, and 2 days longer for organisms treated on day 2,

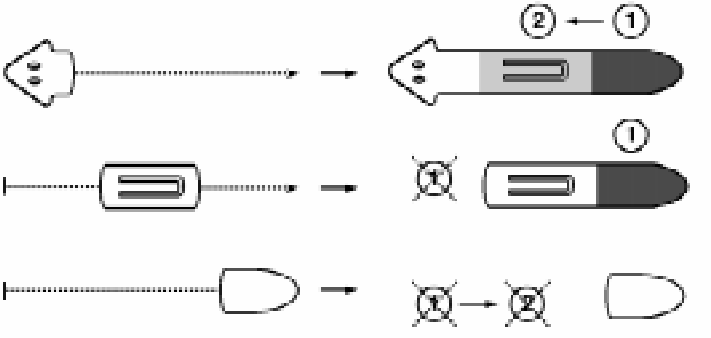

Fig. 4. Diagram showing the disruption of the regeneration process produced by exogenous RA administration and its interpretation according to a disto-proximal sequence of regional determination. **(Left)** *Levels of amputation analysed. Blind-ended lines are for disruption of regeneration. Arrow-ended lines are for normal regeneration.* **(Right)** *Interpretation of the disruption of the regeneration process according to a disto-proximal sequence of regional determination. The old stump is in white; the most distal region to be regenerated in dark grey; and the central region in light grey. (1) and (2) are for the first and subsequent gross molecular regions to be determined during planarian regeneration respectively, according to the proposed disto-proximal model.*

corresponding to the reported regeneration delays after 9 days of regeneration (Fig. 3).

Discussion

Exogenous RA disrupts anterior but not posterior regeneration in planarians

The effects of exogenous application of RA on development and regeneration have been extensively studied in vertebrates. In general, they depend on the doses and the period of administration. For example, RA is essential in the development of the central nervous system and during neuronal differentiation in chicken, *Xenopus* and mouse embryos (Blumerg, 1997); in the patterning of limbs (Tamura *et al.*, 1993; Helms *et al.*, 1996); in the formation of the myocardium in *Xenopus* (Drysdale *et al.*, 1997); in the patterning of the lung in rats (Wellington *et al.*, 1996); etc. In most cases, the effects of exogenous RA can be summarised as the lack of anterior structures or their posteriorisation (the change of anterior to more posterior fates).

Here, the effects of exogenous RA administration during planarian regeneration are similar: disruption of anterior but not posterior regeneration. However, we should elucidate whether this disruption is due to real morphogenetic effects of RA on planarian regeneration (i.e. whether RA is a natural planarian morphogen) or just to toxic effects. There is an intense debate on whether RA is used by invertebrates as a natural morphogen or not. Although the results reported here do not permit to elucidate this controversy, and this is not the aim of this paper, there are several aspects that worth to be mentioned.

First of all, the concentration of RA used in this study was extremely high compared with usual morphogen concentrations. However, the organism is protected by a polysaccharide mucus layer and a relatively strong epithelium (Baguñà, 1973) that efficiently impedes the entry of external substances. So, while we do not know the actual concentration of RA within the animals resulting from the treatment, it was probably just a small fraction of the concentration of the RA in the bath. In this respect, it is interesting to note that organisms treated at day 0 (just after amputation), when the wound was recent and the organism much more accessible to exogenous RA, did not show any defect, in contrast with organisms treated at day 1, when the wound was completely sealed and the barrier that prevent the entering of external substances was rebuilt, suggesting that the reported effects aro not due to a non-specific toxic effect. Moreover, it is possible that the high concentration of RA needed may be due to the hypothetical receptors present in planarians, as retinoid receptors found in other invertebrates (RXR; reviewed by Jones, 1997) do not display high affinity for RA.

Second, whether RA is acting through a morphogenetic or specific toxic effect, it disrupts anterior, but not posterior regeneration. This observation suggests that at least some processes of anterior regeneration differ from those used during posterior regeneration. Third, the duration of sensitivity to RA is very restricted, and coincides with the early events of head determination, at 6-24 hours of regeneration (Saló, 1984; for a general review see Baguñà *et al.*, 1994), suggesting that exogenous RA is disrupting the determination of anterior structures. Moreover, during limb regeneration in vertebrates, the maximum sensitivity to exogenous RA occurs during cell dedifferentiation (Maden, 1997), when cells start to form the regenerative blastema and become determined to form the lost structures. Thus, again whether from a morphogenetic or specific toxic effect, the period of maximum sensitivity in planarian anterior regeneration seems to correspond with the period of maximum sensitivity in vertebrate limb regeneration.

Fourth, the enormous plasticity of planarians allows them to overpass the effects of exogenous RA administration once the treatment ends. This is the reason why animals subjected to a single RA treatment do not definetively arrest anterior regeneration; when the RA treatment ends, the presence of proliferating neoblasts allow the organism to resume regeneration, with the above reported delay.

Although the results reported here do not permit to elucidate whether these effects are due to a morphogenetic activity of the exogenous RA or a response to its toxicity, they are strikingly coincident with the results reported on vertebrates. This makes tempting to speculate that retinoids are natural morphogens in planarians. Specific receptors and molecules involved in the synthesis of molecules belonging to the retinoid pathway should be found in order to understand the effects of exogenous RA activity on planarians.

RA regeneration disruption suggests a disto-proximal sequence of regional determination

The sequence of pattern restoration of several organs and tissues in planarians has been monitored by molecular markers. For example, the formation and restoration of the pharynx follows a proximo-distal, disto-proximal or centro-radial sequence of cell determination/differentiation depending on the cell type or tissue analysed (epithelial, secretory and muscle cells, respectively; Bueno *et al.*, 1997; Kobayashi *et al.*, 1999); the restoration of body wall musculature in the regeneration blastema occurs by intercalation of the new fibres within the old ones (Cebrià *et al.*, 1997); and the regeneration of the brain seems to be disto-proximal (Agata *et al.*, 1998). However, few data are available on the study of the determination of gross body regions by molecular markers. Bueno *et al.* (1996) have reported that the dynamics of TCEN49 localisation during planarian regeneration agrees with a disto-proximal sequence of gross regional determination, in accordance with Wolff's model (Wolff *et al.*, 1964).

The disruption of the process of regeneration by exogenous RA administration also suggests a disto-proximal sequence of gross regional determination. First, the exogenous application of RA inhibits the regeneration of the anterior molecular region into both types of regenerates analysed, derived from amputation at level E or C-D (see Figs. 1 and 4). Second, the regeneration of the posterior molecular region is never inhibited. Third, the regeneration of the central molecular region is inhibited as follows: if the distal contiguous region to be regenerated is the anterior, which never regenerates, the central region does not neither; if the distal contiguous region to be regenerated is the posterior, which always regenerates, the central region does as well (Fig. 4).

This may indicate that, before the determination of the new central molecular region, the distal contiguous region has to be determined. This is in agreement with transplantation experiments (Saló, 1984; for a general rewiev see Baguñà *et al.*, 1994) showing that head determination occurs at 6-24 hours of regeneration, a bit earlier than pharynx determination, which takes place at 12-36 hours of regeneration. Moreover, the period of head determination corresponds to the period of maximum sensitivity to the exogenous RA. These results suggest that, in agreement with Wolff's model (Wolff *et al.*, 1964), the determination of gross molecular regions during planarian regeneration follows a disto-proximal sequence, from the regenerative blastema to the old stump, and that the new central region arises by intercalation within the anterior and the posterior ones, as de-

scribed by the models of positional values intercalation (Maden, 1977, and Slack, 1980).

Materials and Methods

Species, culture conditions and nomenclature

The freshwater planarians used in this study belonged to an asexual race (class A; Ribas *et al*., 1989) of the species *Girardia tigrina* collected near the city of Barcelona. They were maintained in spring water in the dark at 4-6°C and fed once a month with beef liver. The planarians chosen for the experiments were starved for at least 15 days before use. Specimens of 7-10 mm in lenght were cut pre- and/or post-pharyngeally (levels A [head], C-D [trunk] or E [tail], according to Bueno *et al*., 1996; see Fig. 1) and mantained at 17±1°C.

The term blastema designates a small unpigmented mound of tissue made of small undifferentiated (neoblasts) and differentiating cells that form and grow below the wound. Anterior regeneration refers to the process by which planarian fragments regenerate anterior structures; posterior regeneration is the process by which planarian fragments regenerate posterior structures.

RA treatments and immunohistochemistry

Regenerating organisms were treated with exogenous *all-trans*-retinoic acid (Sigma) at a final concentration of 0.5×10^{-3} M in spring water diluted from a 0.1 M stock solution of RA in ethanol (Ferreti *et al*., 1991). Two sets of experiments were carried out. In the first set, animals received daily RA treatments of 2 hours/day during 10 days, from day 0 to 9 after amputation. In the second set, animals received a single 2 hour treatment, at times ranging from 0-7 days after amputation. Organisms were sacrificed at different stages. In both sets of experiments, regenerates were maintained in a 24-well plate (≈5 regenerates/well) with 150 µl/well of RA solution (just to cover the organisms), or in 2 ml of spring water in the meantime. After the 2-hour-treatment, regenerates were washed several times in 2 ml of spring water. Control organisms were treated with the solvent (ethanol) at the same final concentration, in the same volume and during the same period of time. A minimum of 40 specimens were analysed for each condition.

Immunostained sagittal paraffin sections were obtained as described in Romero *et al*. (1991), using the Avidin-Biotin Complex method (ABC, peroxidase conjugated, Vector) to detect the primary antibody (mAb TCEN-49).

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