

Digit regeneration in the amphibian – *Triturus cristatus*

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SUMMARY

Digit regeneration has been examined in *Triturus cristatus*. Because of their size, blastemas that form after digit amputation are relatively a lot more suitable for quantitative studies that involve for example the counting of cells in histological sections. They are also very useful for the study of some basic histological aspects of regeneration particularly cartilage formation. This has been looked at in regenerating digits, as there are only a maximum of three bones to regenerate and these lie in sequence, one after the other. It was seen that the cartilage is laid down as a solid rod by about 17 days post-amputation, and that by about 20 days, it starts to be split up into its three elements.

An X-ray study of the growth of digit regeneration together with autoradiography experiments were also carried out as a comparison to studies already undertaken on larger more proximal blastemas. It was shown that in fact the behaviour of digit blastemas is very similar to those of a more proximal origin. This fact, together with the advantages of its size, make the digit a very strong candidate for the further study of regeneration.

INTRODUCTION

To understand the process of limb regeneration more fully, a complete quantitative picture needs to be built up of several cellular parameters, such as cell cycle time, the number of cells dividing at any one time, the tissue-types of cells undergoing division, etc. This target has been greatly hampered by the tedious experimentation involved in such experiments, which in part can be attributed to the large size of the regeneration blastemas which form upon newt forelimb amputation. This problem could be overcome with the use of small blastemas. With this aim in mind, experiments have been performed on blastemas formed by amputation through the forelimb digits of the newt *Triturus cristatus*.

A histological survey was initially carried out to obtain a reasonable description of digit regeneration. It was hoped that some information might be obtained about the process of cartilage formation, e.g. are the phalanges laid down one at a time or does the cartilage lay down as a single rod and later break up into smaller elements?

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Concomitant with this study, other experiments looking at the rate of growth and the pattern of cell division were also carried out, in the hope that comparable results to those already published for larger blastemas might be obtained (see Smith, Lewis, Crawley & Wolpert, 1974; Smith & Crawley, 1977).

METHODS

Animal care

Adult *Triturus cristatus* of a similar size were used throughout. They were kept in ordinary tap water in heated aquaria (25 °C), and fed twice weekly on minced beef-hearts. All amputations were performed under MS: 222 anaesthesia (1:2000). Digits of the two forelimbs were amputated through the joint of the metacarpal and the first phalanx. The longest digit of each limb was used, this being the second of the digits from the anterior (outside) of the hand. This digit contains three phalanges, whereas the other digits only have two.

Blastemal growth

The digits were left to regenerate normally. At various intervals throughout regeneration the animals were X-rayed and the blastemal length calculated. Details of the method are given in Smith, Lewis, Crawley & Wolpert (1974). Ten digits were measured for 50 days after amputation.

Autoradiography

Animals were injected intraperitoneally at various times after amputation, with 50 μ Ci tritiated thymidine dissolved in 0.05 ml sterile water. This was purchased from the Radiochemical Centre, Amersham. They were left for 2 h before being fixed for autoradiography and histology.

Histology

Blastemas, plus a small piece of stump were cut off the hand after regeneration had proceeded for various periods up to 35 days. These were fixed in half-strength Karnovsky cacodylate-buffered formalin/glutaraldehyde mixture overnight (see Karnovsky, 1965) at 4 °C. The tissue was dehydrated through a graded series of alcohols and embedded in Araldite. Pairs of slides of sections 1 μ m thick were cut at intervals passing longitudinally through the digits. One of the pair of slides was used for autoradiography, the other for histology. Details of the method are described in Smith & Crawley (1977). The slide for autoradiography was stained by the Feulgen method and the slide for histology was stained with 1% toluidine blue (in 1% borax).

RESULTS AND DISCUSSION

Because of the size problems in sectioning large blastemas from amputations made through the upper or lower arm, digit-amputation blastemas were examined to look more closely at the way in which the cartilage is laid down and then split to form recognizable elements. This is easier to study in digit-amputation blastemas because in the longest digit there are only three bones to be replaced and these lie in series one after the other, whereas blastemas from more proximal parts of the arm have the added complication of carpal separation. In the adult digit, muscle is only present in any great quantity up to the end of the metacarpal, though small interphalangeal muscles are present (Grim & Carlson, 1974). Tendons, however, run distally into the digit.

An amputation through the digits, at the metacarpal-first phalanx joint, leads to the formation of a blastema which does not pass through any recognizable stages, based on external criteria (whereas blastemas forming from amputations through the humerus or radius/ulna do). After wound healing a mound forms which simply gets bigger. Therefore these blastemas were staged according to the time after amputation.

Upon amputation, the vasculature of the digit is severed, but blood clotting takes place rapidly, effectively sealing off the ends of the vessels. Vasoconstriction may occur a little later on, as the blood clots can be washed away, yet no more blood is lost. Within a few days, this exposed amputation surface becomes covered by epidermis that has been mobilized and migrates over from the retracted skin. The dermis does not migrate with it. The wound epithelium is thickened to approximately seven to eight cells thick, from the normal three to four cells thick. Histochemical studies by various workers (e.g. Johnson & Singer, 1964) indicate that in the first few days following amputation, the region near to the distal cut surface is anaerobic. This characteristic has been suggested by Revardel & Chapron (1975) and Smith & Wolpert (1975) to be responsible for the breakdown of connective tissue-matrices leading to the release of single cells.

The cells found beneath the apical wound epithelium begin to proliferate, pushing the ectoderm out into a cone-shaped mound, the ectoderm now also dividing to maintain a uniform thickness. About 10 days after amputation are needed for this small conical blastema to form (Fig. 1A). The mesenchyme of these blastemas seems to be composed of a homogeneous mass of cells, visually at least. A few days later, blood vessels are seen entering the blastema. At the same time, mesenchyme cells in the centre begin to flatten slightly at right angles to the main axis of the limb. This is the start of pre-cartilage formation. By days 15–16, differentiation is clearly seen internally with the appearance of a cartilage rod in the central part of the blastema (Fig. 1B). By day 17 the matrix of this cartilage rod stains metachromatically, with equal intensity of stain, from the cartilage of the proximal elements remaining in the stump, right the

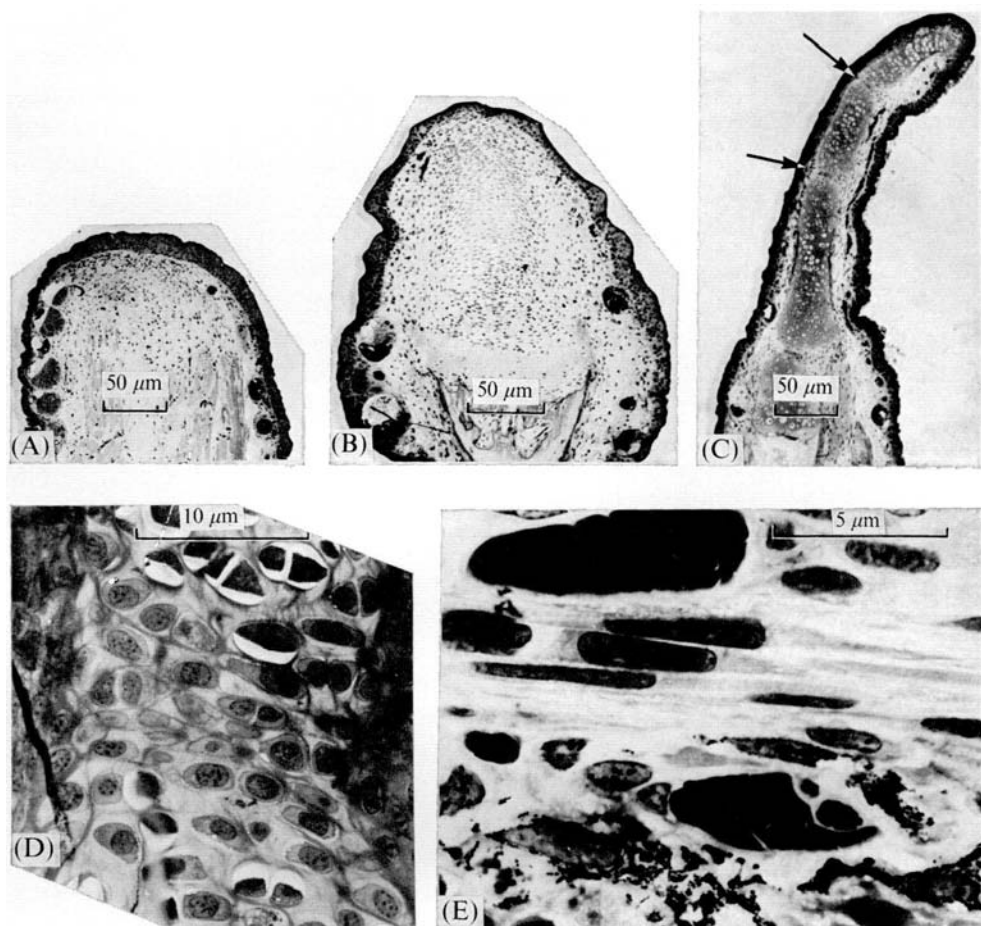


Fig. 1. The histology of digit amputation regenerates. (A) 10-day regenerate – there is a small distal mound of homogeneous cells. (B) 15-day regenerate – the central cells are beginning to stack into pre-cartilage rods. (C) 35-day regenerate – all three phalanges have regenerated (arrows indicate joint regions). (D) Part of the cartilage rod of a 20-day regenerate, showing the alternating regions of hypertrophy. The central region is the future joint. (E) Elongated cells in the lateral parts of a 20-day regenerate.

way along the element to its distal limit. Changes in the staining properties of the cartilage matrix, along the length of the rod, are apparent by about 20 days. The rod now shows the appearance of alternating darkly stained regions with others of much lighter stain (Fig. 1C). The lighter-staining regions have very compact cells which fill the entire space of their own individual lacunae, whereas the darker-staining regions have chondrocytes spaced much further apart and the cells only occupy about half of the available space in their individual lacunae. They appear to be undergoing hypertrophy, this being the forerunner to bone deposition in endochondral ossification. These events are all prior to

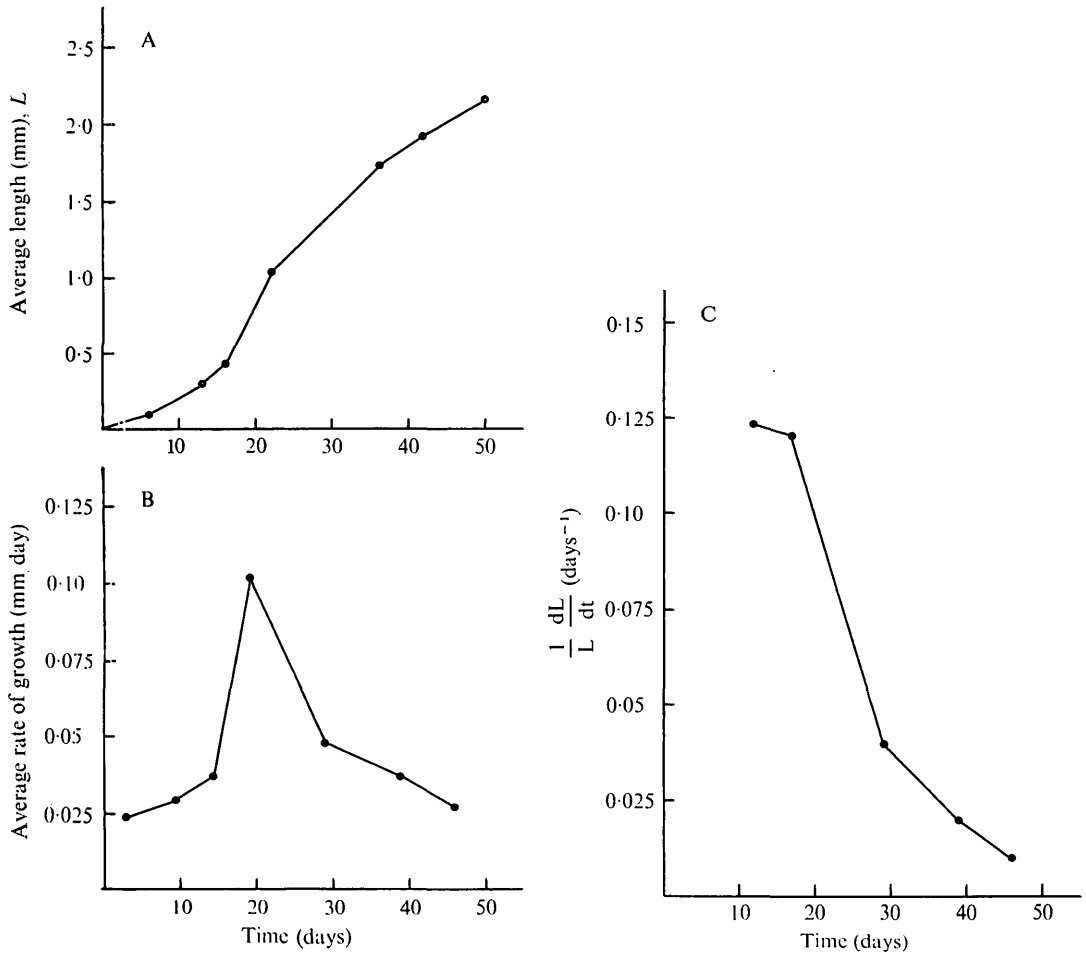


Fig. 2. The growth curves of digit amputation regenerates. (A) The change in length (L). (B) The rate of elongation (dL/dt). (C) The intrinsic growth rate ($[dL/dt]/L$).

joint appearance. Between days 20 and 27, proper joints are seen in the more darkly staining regions, i.e. the separation of cartilage cells and matrix into two distinct elements (Fig. 1D).

Tendon regeneration also occurs after amputation. Beginning at about 17 days after amputation, flattened cells, elongated along the axis of the limb, are observed (Fig. 1E). Electron microscopy shows these cells to be fibroblasts secreting collagen. The appearance of these fibroblasts is well established by day 25 post-amputation. By day 35, bone is starting to be laid down.

It therefore appears that the cartilage is laid down as a solid block and then broken up into the separate phalanges. This is similar to the description of carpal formation by Stocum & Dearlove (1972). They examined *Ambystoma*

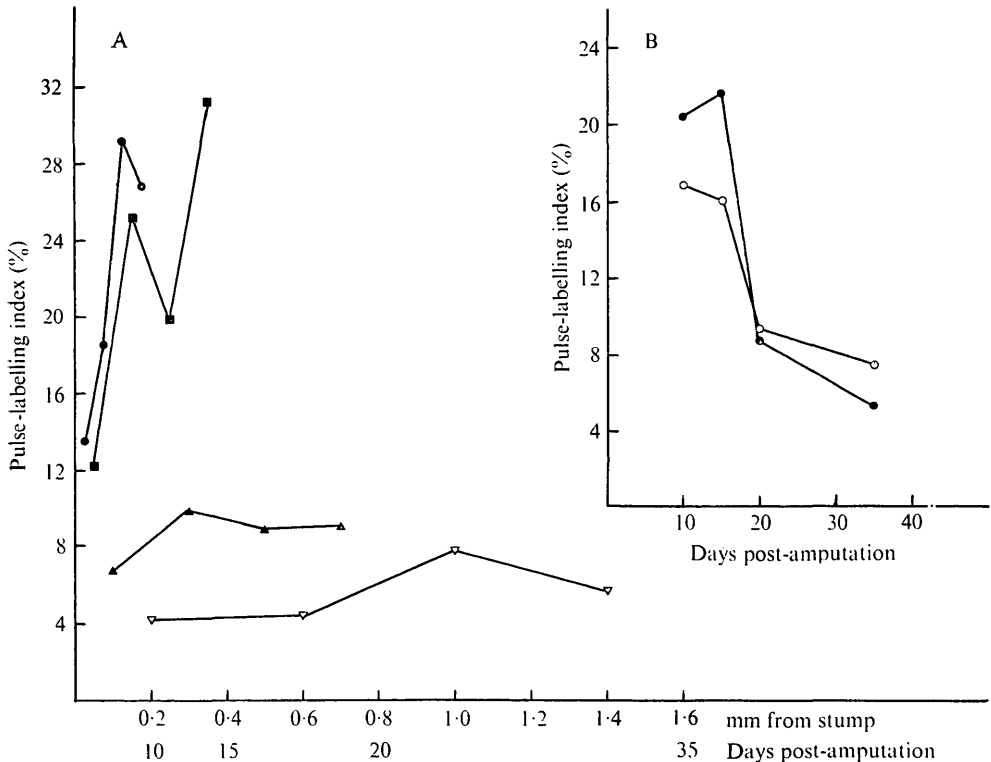


Fig. 3. Results of pulse-label autoradiography of digit amputation regenerates. (A) Regenerates at 10 (●), 15 (■), 20 (▲) and 35 (▽) days were divided into four equal segments parallel to the plane of amputation. The pulse-labelling index for each segment was calculated and plotted as distance from stump for the various blastemas. The overall length of the 10-, 15-, 20- and 35-day regenerates is indicated. For all regenerates, the distal half pulse-labelling index is higher than the proximal half. (B) Pulse-labelling index of the whole blastema ectoderm (○) and mesoderm (●) plotted against time. In both cases there is a rapid drop between days 15 and 20.

blastemas *in vitro*, and also found that cartilage was first laid down as a block and later split and modelled into sub-units.

An X-ray study of the rate of regeneration was also undertaken using ten individuals. The results are shown in Fig. 2, and are remarkably similar to those obtained for more proximal large blastemas (Smith *et al.* 1974). That is, a typical sigmoid growth curve is seen (Fig. 2A) which can be divided into three phases: (i) a period of slow growth during which the blastema is formed-up to about day 12, (ii) a period of exponential growth when differentiation takes place – up to about day 30, and (iii) a final period of slower growth, as the newly regenerated elements reach their fully regenerated length. It can be seen that the period of maximum rate of growth occurs between days 16 and 22 (Fig. 2B). Histologically, this is when the cartilage rod is splitting up into its elements.

The intrinsic growth rate ($[dL/dt]/L$) (Fig. 2C), – that is, the change in rate of growth per unit length – gives some information about the behaviour of the ‘unit cell’ in the regeneration blastema. It is interesting to note that the curve obtained for digit regenerates appears to be identical to those obtained for humerus, radius/ulna and carpal regenerates, as described in Smith *et al.* (1974), even though the blastema here examined is quite different to the more proximal ones in both size and make-up (there is no muscle present). This indicates that the cells in the blastemas from all four levels of amputation are behaving in a similar fashion. As shown in that paper, the maximum intrinsic growth rate continues for slightly longer in the more proximal regenerates, producing bigger primordia, the more proximal the level of amputation. This period, however, is only a small part of the regeneration time, the main part being the growth of these rudiments. This means that the total regeneration time for any level of amputation is very similar. Indeed, as long ago as 1768, Spallanzani reported that the regeneration of a salamander toe took as long as a whole leg.

The results of the pulse-label autoradiography experiments are shown in Fig. 3. At least two animals at days 10, 15, 20 and 35 post-amputation were used. For counting purposes, blastemas were divided into four equal parallel strips running horizontally across the blastema. The highest labelling index occurs at 10–15 days, when the blastema is still forming (Fig. 3A). This is just prior to the period of maximum growth, which seems more than likely to be due to cartilage matrix secretion than cell division. The labelling index of the whole blastema in both the ectoderm and mesoderm can be seen to fall with time (Fig. 3B).

All blastemas had a higher labelling index in the distal half than the proximal half, but the gradient was not as smooth as that seen in the radius/ulna regenerates described in Smith & Crawley (1977). The results obtained in the present paper are not directly comparable to those of the previous paper as different animals (size and age) were used at a different time of year. All of these variables have been shown to affect growth (see Schmidt, 1968).

Summarizing the results it can be seen that the highest pulse-labelling index occurs between days 10 and 15 and the highest growth rate occurs between days 16 and 22. From histology, it was shown that cartilage forms at about 15 days but does not break up into phalanges until about 20–27 days. Thus it would appear that the blastema forms a mass of cells by 10–15 days which begin to differentiate as cartilage, tendon, etc., almost immediately. The chondrocytes begin to secrete matrix leading to the period of maximum growth by about 20 days. It is only after this that secondary signs of differentiation appear, i.e. the cartilage rod splits into two. The same might be true for muscle re-differentiation in more proximal regeneration blastemas.

According to the progress-zone model of regeneration (Smith *et al.* 1974) cells of the early blastema are assigned positional values which determine their future differentiation. From the results reported it would appear that the

interpretation by the cells of these internal records occurs in a step-like manner; the first step being a coarse one and the secondary step a fine adjustment.

It therefore appears that blastemas formed by amputation through digits behave in the same manner as those forming from amputations through more proximal parts of the limb. The very nature of the size of the digit blastema, however, could make it a much more suitable candidate for the further study of regeneration.

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