The regeneration of limbs in adult anurans

By ROBERT P. GOODE

From the Department of Zoology, Columbia University, New York

INTRODUCTION

It is generally accepted that among the amphibians only urodeles and larval anurans retain the power to regenerate limbs and digits. However, there have been reports of atypical limb regeneration in individual post-metamorphic frogs occurring spontaneously in nature and in the laboratory after simple amputation (reviewed by Vorontsova & Liosner, 1960). On occasion, atypical limb regeneration has been observed in a few lizard specimens (Hellmich, 1951; Kudokotsev, 1960; Poyntz & Bellairs, 1965).

In 1932 Rostand found that recently metamorphosed individuals of the African ‘clawed toad’, Xenopus laevis, consistently gave ‘heteromorphic’ regenerates upon amputation. Such growths will be designated here as ‘hypotypic’ according to the criteria used by Needham (1952). Similar observations were reported by Beetschen (1952), Gitlin (1956) and Skowron & Komala (1957), while Komala (1957) and Dent (1962) traced the ontogenetic development of regenerates in larval and metamorphosing individuals.

A high incidence of limb regeneration after simple amputation in adults of other anuran species has been reported, namely Bombina bombina and Rana temporaria, Polezhaev (1946, 1947) and Hymenochirus boettgeri, Goode (1962), but as yet no detailed histological studies have been made. The present study reports a histological analysis of regeneration in several species of adult anurans.

MATERIALS AND METHODS

Representatives of two anuran families were examined—Pipidae and Discoglossidae. The Pipidae used in this study were early post-metamorphic Xenopus müller, adult X. laevis, and adult Hymenochirus boettgeri. The Discoglossidae examined were adult Bombina bombina, B. variegata, and Discoglossus pictus.

Amputations were performed by cutting midway between elbow and hand, or knee and foot, with scissors.

At various intervals limbs were fixed and decalcified in Bouin’s solution for...
periods of up to 2 months, depending on the size of the specimen. Material was dehydrated and cleared in cellosolve and amyl acetate, embedded in paraffin, and sectioned serially at 10 μ. The tissues were stained with hematoxylin and counterstained with eosin or orange G. Selected material was stained for connective tissue with Van Gieson’s picrofuchsin, Masson’s trichrome or Mallory’s anilin blue collagen method, and for nerve fibers with silver nitrate by the method of Palmgren (1960).

RESULTS

A. Gross observations

The percentage of regenerates obtained varied with the species examined (Table 1). Although the pattern of limb regeneration was highly variable, there were features common to all species studied. Development usually proceeded in four stages:

1. Wound closure. This period began soon after amputation and was usually completed by the second or third day, although complete closure was sometimes delayed for weeks by protruding bone.

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of limbs amputated between elbow and hand (or foot)</th>
<th>No. in which regeneration observed</th>
<th>Regenerates obtained (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Xenopus mulleri</em> (early post-metamorphic)</td>
<td>20</td>
<td>20</td>
<td>100.0</td>
</tr>
<tr>
<td><em>X. laevis</em> (adult)</td>
<td>65</td>
<td>63</td>
<td>96.9</td>
</tr>
<tr>
<td><em>Hymenochirus boettgeri</em> (adult)</td>
<td>52</td>
<td>45</td>
<td>86.5</td>
</tr>
<tr>
<td><em>Discoglossus pictus</em> (adult)</td>
<td>30</td>
<td>24</td>
<td>80.0</td>
</tr>
<tr>
<td><em>Bombina variegata</em> (adult)</td>
<td>36</td>
<td>25</td>
<td>69.4</td>
</tr>
<tr>
<td><em>B. bombina</em> (adult)</td>
<td>34</td>
<td>21</td>
<td>61.7</td>
</tr>
</tbody>
</table>

2. Period of demolition. The limb developed a swollen, ‘clubbed’ aspect. This period usually extended through the third or fourth week, and sometimes into the sixth week after amputation. The end of this period was marked by the appearance of a distinct edematous swelling at the tip of the limb (pre-bud stage).

3. Regeneration bud. An opaque bud, or blastema, first became evident 1 or 2 weeks after the end of the preceding period. In a number of cases no regeneration bud was observed. Although migrating epidermis covered all amputational wounds, and all stumps underwent a period of inflammation and demolition, some did not regenerate. In these cases the wounds were eventually sealed with stump skin and exhibited only callus and scar formation.
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4. Later development of the regenerate. The bud increased in length at a constant rate. In some cases growth ceased after a few weeks, while in others it continued for several months. Regenerates ranged from short, spatulate growths to long, thin protrusions, occasionally with paddle-like distal ends and rudimentary digits. A few limbs approached the normal in length but none in extent of distal morphogenesis (Plate 1, figs. 1–4).

B. Histological observations

All histological observations discussed are of forelimbs amputated midway between elbow and wrist.

Wound closure. During the first few days after amputation, epidermal cells from the edges of the wound migrated over the surface of amputation. The dermis and basement membrane, however, remained at the edges of the wound. The resulting wound cover thickened, chiefly by cellular migration, but rarely attained a size comparable to that of the apical caps observed in regenerating urodele limbs by Schmidt (1958).

Period of demolition. Cells liberated from the marrow cavity and injured periosteal sheath accumulated beneath the wound epidermis. Large numbers of blood cells and fragments of stump tissues also were observed. The whole region appeared swollen and edematous. Long tongues of epidermal cells extended deep into areas of degradation, recalling the ‘graveyard activity’ of epidermis described by Singer & Salpeter (1961).

As histolysis of injured tissues proceeded, multinucleated giant cells appeared in small numbers. These cells were found at the tip of the bone, along its shaft, and surrounding bone fragments lying in subepidermal regions (Plate 1, figs. 5, 6).

Extensive morphological changes were observed in muscle of the stump. These included disappearance of cross-striations, dissolution of myofibrillae and rounding up and enlargement of nuclei. These changes resembled the alterations in structure of muscle accompanying histolysis in regenerating limbs of urodeles and tadpoles (Schmidt, 1958; Dent, 1962).

Concomitant with histolytic changes in the tissues of the stump was a several-fold increase in thickness of the periosteal sheath subsequent to amputation. At the end of the period of demolition, centers of cartilage formation were seen in the thickened periosteum. These centers eventually fused, producing a collar of cartilage which usually extended for a short distance proximal to the end of the bone (Plate 1, fig. 5).

Pre-bud stage. As a result of histolytic activity during the period of demolition, much of the projecting stump bone and tissue debris had been eliminated from the wound area. Masses of cells, occupying a roughly crescent-shaped area distal to the end of the intact shaft and periosteal collar, were found in the wake of the histolysed bone (Plate 1, fig. 7). This region of accumulation consisted of marrow and blood cells, osteoblast-like cells probably liberated
from the bone and its investing sheaths, and large numbers of multinucleated giant cells. The giant cells were often associated with persisting fragments of bone, and were also found free in the wound area, surrounded by mononucleate osteoblast-like cells (Plate 2, fig. 8). Sometimes giant cells were massed in large numbers beneath the epidermal wound cover.

Small areas of young cartilage were found in the region of cellular accumulation distal to the bone. These chondrogenic centers became increasingly prominent as development proceeded.

Fibroblast-like cells, which appeared to originate chiefly from the region of muscle and connective tissue lateral to the bone, were found in small numbers beneath the wound cover.

*Regeneration bud.* The pattern of regeneration after the preceding stage was highly variable. By the time a distinct regeneration bud was first visible most of the wound area was free of bone, except for small fragments that occasionally persisted well into later stages of regeneration.

The region immediately distal to the intact bone shaft and periosteal collar was in an advanced stage of chondrification and often formed a distinct cap or bridge over the tip of the bone.

A distinct region of fibroblast-like cells and associated fibers was found distal to the periosteal collar and cartilage cap of the bone. Chondrogenesis was prominent within this blastemal accumulation (Plate 2, figs. 9, 10). The fibrocellular mound was penetrated by large numbers of nerve fibers (Plate 2, fig. 11).

*Later development of the regenerate.* Sometimes the definitive regenerate was short and spatulate and consisted chiefly of a thick, irregular mass of cartilage, with a dense fibrous connective tissue cap. Most frequently, the regenerate

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**Plate 1**

Fig. 1. Hind limb regenerate of adult *Xenopus laevis.* Note the developing foot and digital buds. ×3.

Fig. 2. Forelimb regenerate of *Bombina bombina.* ×6.

Fig. 3. Forelimb regenerate of *Hymenochirus boettgeri.* ×5.

Fig. 4. Forelimb regenerate of *Discoglossus pictus.* ×5.

Fig. 5. Forelimb regenerate of *B. bombina* 14 days after amputation. Small osteoclasts may be seen around the tip of the stump bone. The thickened periosteal sheath is chondrifying. Note the tongues of epidermis surrounding a large piece of bony debris. o, Osteoclast (giant cell); p, periosteal cartilage; t, epidermal tongue. H. and E. ×100.

Fig. 6. Splinter of bone in the tissues between the wound epidermis and the stump bone in a forelimb of *Bombina variegata* 20 days after amputation. Note the osteoclasts surrounding the splinter. H. and E. ×400.

Fig. 7. Forelimb regenerate of *D. pictus* 30 days after amputation. Note the chondrified periosteal collar and the large focus of cartilage in the cellular accumulation. A small accumulation of fibroblast-like cells lies immediately beneath the epidermal wound cover. c, Cartilage; p, periosteal cartilage. H. and E. ×100.
Fig. 8. High-power view of cells distal to the bone in the regenerate shown in fig. 7. Note the multinucleated giant cells. a, Osteoclast (giant cell). H. and E. x 1000.

Fig. 9. Distal part of a regenerating forelimb of adult *Xenopus laevis* 48 days after amputation. The blastemal accumulation can be seen beneath the epidermal wound cover. b, Blastemal cell. H. and E. x 100.

Fig. 10. Distal part of a regenerating forelimb of *Bombina bombina* 56 days after amputation. Note the blastemal accumulation beneath the epidermis. Proximally, procartilage can be seen. b, Blastemal cells. H. and E. x 100.

Fig. 11. Distal tip of a young *X. mulleri* forelimb 25 days after amputation. Note the nerve fibers penetrating the blastema. Silver nitrate and orange G. x 120.

Fig. 12. Definitive forelimb regenerate of *B. bombina*. The cartilage rod is cut through in several places by connective tissue. H. and E. x 40.

Fig. 13. Regenerated muscle slips in dense connective tissue of the proximal part of a forelimb regenerate of *X. laevis*. m, Regenerated muscle slip. Masson’s Trichrome. x 400.

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Fig. 14. Healed forelimb stump of *Hymenichirus boettgeri*. The periosteal cartilage and cartilage cap are well formed. The area distal to the cartilage is filled in by dense fibrous scar tissue. Van Gieson's stain. ×40.

Fig. 15. Developing cartilage within the fibrous scar of a healing *Discoglossus pictus* forelimb. c, Cartilage. H. and E. ×400.

Fig. 16. High-power photomicrograph of the blastema in the *Xenopus laevis* forelimb shown in fig. 9. Cells are small and fibrocyte-like, with much intercellular fiber. H. and E. ×400.

Fig. 17. High-power photomicrograph of the blastema in the *Bombina bombina* forelimb shown in fig. 10. Cells are large, and the blastema is sparsely fibrous. H. and E. ×400.

Fig. 18. Oil-immersion view of the blastema shown in fig. 17. Note the round cells with large nuclei and cytoplasmic processes. H. and E. ×1000.
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was long and thin, and consisted of a rod of cartilage surrounded by connective tissue (Plate 2, fig. 12). In a few cases the cartilage was cut through in several places by connective tissue, and occasionally was separated from the bone stump and its cartilage cap by fibrous scar tissue.

Regeneration of dermis was usually delayed until cartilage development was under way. New dermis developed proximo-distally from fibroblasts under the epidermal wound cover. However, dermal connective tissue did not invade the growing tip of the regenerate.

Regeneration of muscle was limited to a few small striated slips continuous with the muscle of the stump. Often the muscle fibers were almost indistinguishable from the abundant fibrous connective tissue lateral to the cartilage of the regenerate (Plate 2, fig. 13).

In cases in which regeneration did not occur thick connective tissue developed within subepidermal regions. Although histolysis, demolition, and release of cells from stump tissues were observed, regeneration was limited to precocious chondrification of cells in the vicinity of the bone, and cicatrization of more distal regions (Plate 3, fig. 14). On occasion, a focus of cartilage formation was found within the developing scar tissue (Plate 3, fig. 15).

There were differences among the anurans studied in the rate of regeneration and the characteristics of the regenerate. There was a relatively longer period of demolition and a relatively greater development of periosteal collar and connecting cartilage bridge in *Xenopus* and *Hymenochirus* than in *Bombina* and *Discoglossus*. In *Xenopus* and *Hymenochirus* the blastemal cells were small and spindle-shaped and were interspersed with a considerable number of intercellular fibers (Plate 3, fig. 16). Sometimes the blastema closely resembled fibrocellular connective tissue. In *Bombina*, and to a lesser degree in *Discoglossus*, the blastemal cells were larger and rounder, with extensive cytoplasmic processes, and were only lightly interspersed with fibers (Plate 3, figs. 17, 18). However, the cell density and frequency of observed mitotic figures were low in all species studied. Muscle regeneration was more prominent in *Bombina* and *Discoglossus*, while the percentage and relative length of regenerates was higher in *Xenopus* and *Hymenochirus*.

**DISCUSSION**

This study demonstrates extensive regenerative responses in six species of adult anurans, including three in which limb regeneration previously had not been reported—*Xenopus mulleri*, *Bombina variegata* and *Discoglossus pictus*. In addition, the present results do not support the conclusion of Polezhaev (1946, 1947) that adult *Bombina bombina* of similar age and size to those studied here no longer regenerate.

Observations indicated a great similarity in pattern and incidence of regeneration between *Xenopus* and *Hymenochirus*. Similarly, the regenerative processes
in *Bombina* and *Discoglossus* resembled each other more closely than they did the other two genera studied. This is perhaps a reflection of the phylogenetic relationships existing among these animals. *Xenopus* and *Hymenochirus* belong to the family Pipidae, while *Bombina* and *Discoglossus* belong to the Discoglossidae.

A prominent characteristic of regenerates, consistently observed in this study, was the prolonged histolysis of bone and the profusion of multinucleated giant cells, or osteoclasts (Schmidt, 1958) in the wound area throughout the period of demolition. While the function of these cells has been questioned (reviewed by Hancox, 1956), there is little doubt that they play a role in the histolysis of bone in limbs of the species examined in the present study. Their early appearance and close association with the degraded bone of the stump suggest a histolytic function. These giant cells were also found in large numbers in the chondrifying region of accumulation distal to the bone, well past the period of active histolysis. In the light of this observation it would be valuable to investigate their possible role as a source of cells for the cartilage of the regenerate. That giant cells play such a role in amphibian limb regeneration has been suggested in the literature (Hellmich, 1930; David, 1934; Ide-Rozas, 1936).

The cartilage of the regenerate in amphibian limbs seems to be derived from three major sources (see also Wendlestadt, 1910). The earliest to appear is the thickened periosteal component, resembling the early response of bone to fracture described by Pritchard & Ruzicka (1950). The second component seems to arise chiefly from cells released from the bone and its investing sheaths by amputation and histolytic activity of giant cells. This region of cellular accumulation develops chondrogenic centers which join the periosteal collar forming a cap over the end of the bone. In those anurans in which only wound healing occurred the development of a periosteal collar and cartilage cap was the full extent of the skeletal response to amputation and resembled the development of callus after amputational wound healing in lizards and mammals (Pritchard & Ruzicka, 1950; Schotte & Smith, 1959). The third, and most important, component of the skeletal regenerate in amphibian limbs is the mound of mesenchymatous cells, or blastema, accumulating beneath the epidermal wound cover. This component produces in tadpoles and adult urodeles a complete, or nearly complete model of the missing skeletal parts (Forsyth, 1946; Schmidt, 1958). In the anurans studied here a blastema did form. However, unlike the tadpole and urodele blastema, it was relatively diffuse and reduced in mitotic activity. In studies on regenerating limbs of late pre-metamorphic and early post-metamorphic *Xenopus*, Skowron & Komala (1957) noted the formation of a diffuse, ephemeral 'pseudoblastema', consisting of darkly staining cells and fibrous elements. However, they attributed regeneration solely to a process of direct regrowth of stump tissues without the intervention of a distinct blastema. The present study suggests that this 'pseudo-
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blastema’ is, in fact, the major source of cells for the cartilage of the regenerate (see also Dent, 1962; Lecamp, 1963).

All the regenerates examined in the present study were morphogenetically deficient. They were characterized by the production of a blastema reduced in size, and the development of considerably greater quantities of cartilage and connective tissue than muscle. Where only wound healing took place, callus and scar tissue rapidly developed, and muscle regeneration was limited to a few fibers within the scar. There are reports that precocious formation of cartilage and connective tissue in regenerating limbs of tadpoles and post-metamorphic anurans may result from inductive influences emanating from stump tissues (Gidge & Rose, 1944; Lecamp, 1955, 1960). It is possible that the persistent influence of stump tissues, particularly dermal connective tissue and bone not removed by histolysis, may be a factor in the disturbance of normal tissue relations in hypotypic regenerates, and the complete suppression of limb regeneration during amputational wound healing. Increasing the speed and extent of removal of stump tissues would inhibit their influence and might lead to conditions more favorable to complete regeneration. While mechanical removal of bone from the stump is incapable of stimulating regeneration in higher vertebrates, increasing its histolytic elimination has been successful (Umanski & Kudokotsev, 1951, 1952).

SUMMARY

1. The regeneration of limbs, after simple amputation, in two families (four genera) of primitive anurans was investigated. All produced hypotypic regenerates of varying size and degree of morphogenesis.

2. Despite certain differences possibly related to phylogenetic affinities, both Discoglossidae and Pipidae regenerated in similar fashion.

3. Regenerates consisted chiefly of well-developed rods of cartilage, surrounded by connective tissue and skin. Muscle regeneration was minimal, resulting in the formation of small striated slips.

4. The role of the stump tissues and their possible relation to the development of the hypotypic regenerate was discussed.

RÉSUMÉ

1. On a étudié la régénération des membres, après amputation simple, chez deux familles (quatre genres) d’Anoures primitifs. Tous ont produit des régénérats hypotypiques dont la taille et le degré de morphogenèse étaient variables.

2. En dépit de certaines différences, peut-être en rapport avec des affinités phylogénétiques, les Discoglossidae et les Pipidae ont régénéré de manière semblable.
3. Les régénérats consistaient principalement en baguettes de cartilage bien développées, entourées de tissu conjonctif et de peau. La régénération des muscles était minime, avec formation de petites bandes striées.

4. On a discuté le rôle des tissus du moignon et leurs rapports éventuels avec le développement du régénérat hypotypique.

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REFERENCES


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