Forelimb Regeneration in the Larvae of the African Clawed Toad (*Xenopus laevis*), with Special Reference to the Developmental Stage of the Animals[1)]

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(With 1 Table and 1 Plate)

It has been accepted that in anuran Amphibia the ability to regenerate amputated limbs is generally manifested only during the larval period. The developmental stage at which the ability is lost, however, varies among the individual animals of the same species as well as between different species (Liosner, 1931; Borssuk, 1935; Forsyth, 1946). Schotté and Harland (1943), by cutting at different levels the hindlimbs of *Rana clamitans* tadpoles in a certain developmental stage, found that the regenerative potency was lost first in the proximal portion of the limb and, then, progressively in the more distal portion. This result corresponds to the fact that in the anuran limb bud differentiation of component tissues proceeds in proximo-distal direction. Also, Dent (1960, 1962), cutting the hindlimb buds of *Xenopus laevis* at different developmental stages, found that the number of regenerated digits decreased as the larvae developed.

The present study was undertaken to clarify the relationship between histogenesis and regenerative potency of the forelimb in *Xenopus laevis*.

Materials and Methods

The larvae of *Xenopus laevis* used in this study developed from the eggs obtained by injection of chorionic gonadotropin. All the animals were maintained in aquaria kept from 20°C to 22°C, and fed on alfalfa powder throughout the larval stage. Their developmental stage was determined according to the normal table of Nieuwkoop and Faber (1956). For this experiment, only the larvae from stage 52 to stage 61 have been used. Prior to amputation, anesthesia was made in MS 222 (Sandoz) solution (1:4000 in concentration). Amputations were performed through the middle portion of right limb buds with a pair of iridectomy scissors. The middle portion of limb buds from the larvae at stages 56 to 61

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corresponds internally to the portion of differentiating radio-ulna. The left unoperated limb buds served as controls. The amputation of the limb buds was made on approximately 40 larvae at each developmental stage.

For histological study one or two animals were fixed in Bouin's fluid at 2 to 5 days for 20 days after amputation during the phases of regenerative processes. Remaining animals were allowed to grow at least up to stage 61 in order to see the number of regenerated digits. Histogenesis of forelimbs was studied with the right limb buds of the operated larvae and the right forelimb of intact larvae, both fixed in Bouin's fluid. Ordinary serial paraffin sections were cut at 8μ in thickness and stained with Heidenhain's Azan stain. Some sections were treated with 0.05% toluidine blue (citric buffer at pH 4.2) for the purpose of determining the time of appearance and disappearance of metachromasia in the cartilaginous matrix.

Observations

I. *Histogenesis of the forelimb*

At stage 53, forelimb buds externally became paddle-shaped. Many mitotic figures of the mesenchymal cells were seen (Fig. 1). In the larvae at stage 54, a compact mass of mesenchymal cells began to differentiate into cartilage in the proximal portion. An intercellular substance of these mesenchymal cells exhibited metachromasia with toluidine blue (Fig. 2).

At stage 55, the differentiation of cartilage advanced to the distal portion. In some regions of the buds, the arrangement of mesenchymal cells became relatively loose where these cells, originally spindle-shaped, were transformed to oval in shape. At stage 56, elbow and wrist were clearly recognized externally. The boundary between the cartilage and other mesenchymal cells became clear. The nucleus of some mesenchymal cells was surrounded by a thin, acidophilic cytoplasm. These cells were presumably under the process of differentiation into muscle, because the striations began to appear in them at stage 57 (Fig. 3).

During the period from stages 57 to 58, differentiation of all the cartilaginous components proceeded so greatly of the limb that humerus, radio-ulna, carpus and phalanx were clearly distinguished. At stage 61, the myoblasts were in contact with perichondrium of the cartilage. At least histologically, the larvae at this stage had both cartilages and muscles differentiated almost as equally as those in adults.

II. *Experimental results*

Amputation at stages 52 and 53. The wound surface was covered by migrating epidermis within a few hours. Epidermal cells were piled up in the center of the wound surface to form a "tongue" as described by Forsyth (1946) and Dent (1962). Then the wound epidermis became thicker to form an apical cap (Fig. 4). No difference was found between these cells and mesenchymal cells of the limb bud.

Five days after amputation, cells that were undergoing differentiation into either cartilage or muscle were not observed yet in operated limb buds. Growth

Table 1. Differentiation of limb buds in *Xenopus* larvae amputated at various stages of development through the middle portion of the limb buds

Developmental stage of the larvae when amputation was performed	Number of regenerated limb buds with:				Total
	4 digits	3 digits	2 digits	1 digit*	
52	28	0	0	0	28
53	12	0	0	0	12
54	17	9	1	1	28
55	6	22	6	1	35
56	0	8	6	0	14
57	0	2	12	3	17
61	0	0	0	14	14

* The limb buds having differentiated no digits at all were not discriminated from those having already differentiated one digit.

of limb bud depended mainly on the multiplication of mesenchymal cells. Complete four digits differentiated (Table 1). The histogenesis and growth in operated limb buds were the same as those in intact limb buds.

Amputation at stage 54. Three days after amputation, differentiation of the cartilage was seen in the middle portion of the stump, but no blastema cells were derived from this cartilage and the cartilaginous matrix was still metachromatic in nature. It is interesting to note that the regenerated digits decreased in number as the amputation was made at stage 54 when the formation of cartilage began in the limb buds.

Amputation at stage 55. Two days after amputation, the first blastema cells that were originating from the cartilage were observed in the distal portion and the cartilaginous matrix changed its staining property (Fig. 5). The first appearance of blastema cells in the limb buds amputated at this stage and the peak of the occurrence of three digits in the regenerate coincided (Table 1). Seven days after amputation, the buds grew and differentiation of the radio-ulna further proceeded, and dedifferentiation of the cartilage was not seen.

Amputation at stage 57. Two days after amputation, another type of blastema cells with nucleoli, stained with toluidine blue or azocarmin, was observed to originate from dedifferentiating muscle (Fig. 6) and migrated toward the distal portion. Appearance of these blastema cells derived from muscle cells in the buds amputated at stage 57 and the highest occurrence of two digits in such regenerates (Table 1) seemed more than coincidental. As the cartilaginous matrix became gradually non-metachromatic, an oval-shaped nucleus of blastema cell manifested one distinct nucleolus stained by toluidine blue (Fig. 7). Ten days after amputation, the blastema cells started to differentiate into new cartilage

around the perichondrium of old cartilage, while dedifferentiation of the cartilage and muscle was under way in the proximal portion at the same time.

Amputation at stage 61. Blastema cells that had originated from cartilage and muscle accumulated beneath the apical cap of the wound epidermis (Fig. 8). All the operated limb buds assumed the form of a heteromorphic spike like the one produced in adult specimens (Table 1). The spike consisted of cartilage and little or no muscle.

Discussion

Earlier investigators have considered that "dedifferentiation" of tissues was a necessary step in the regenerative process in amphibians (Butler, 1933; Thornton, 1938). The lack of dedifferentiation in adult anurans in general led to failure of regeneration (Rose, 1944; Gidge and Rose, 1944). Also, Rose (1942, 1944, 1945) found that the application of saturated sodium chloride solution to the wound was effective in eliciting limb regeneration in adult frogs. These results seem to confirm the view that the dedifferentiation of tissues is initially an active phase in the regenerative process of Amphibia.

In the adult newt, *Triturus viridescens*, the highest mitotic frequency was observed during the dedifferentiation stage of regenerating forelimbs (Manner, 1953; Chalkley, 1954, 1959). Hay and Fischman (1961) found in the same species that DNA synthesis proceeded most actively in dedifferentiating tissues, and suggested that the dedifferentiation of tissues was an essential and active process in the early phase of regeneration. In the present study, it was indicated that the number of digits decreased as the amputation was performed on older larvae of *Xenopus*. The mesenchymal cells have an intrinsic potency to differentiate into digits while the blastema cells have an intrinsic potency to regenerate heteromorphic spike. It may be concluded, therefore, that it is the quantitative correlation between the two types of cells contained in the regenerates that determines the attaining degree of regeneration.

Summary

In *Xenopus* larvae at stages 52–61, forelimb buds were amputated at the level of the middle part. Differentiation of four complete digits, which took place when the amputation was made on young larvae (stages 52 and 53), was brought about by multiplication of mesenchymal cells. The number of regenerated digits decreased as the amputation was made on older larvae (stages 54–57), showing apparently that the regenerative potency in forelimb buds is in direct proportion to the number of mesenchymal cells and is in inverse proportion to the number of already differentiated cells in the buds.

Acknowledgments

The author wishes to express his cordial thanks to Professor Tomoji Aoto for his continued guidance and careful revision of the manuscript. He is also indebted to Professor Yasuhiko Kanoh for his kindly providing the author with the opportunity to complete this manuscript.

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Explanation of Plate VI

Figures 1–3. Sections of the forelimb buds of larval *Xenopus* to show their normal histogenesis.

Fig. 1. A forelimb bud from a larva at stage 53. Note the occurrence of many mitotic figures (arrows) in mesenchymal cells. Azan. ×400.

Fig. 2. A forelimb bud of a larva at stage 54, showing differentiation of cartilage with the intercellular substance that exhibits metachromasia. Toluidine blue. ×400.

Fig. 3. A forelimb bud of a larva at stage 57, showing differentiation of muscle cells with clear striations. Azan. ×1000.

Figures 4–8. Sections of the regenerates developed from amputated forelimb buds of larval *Xenopus*.

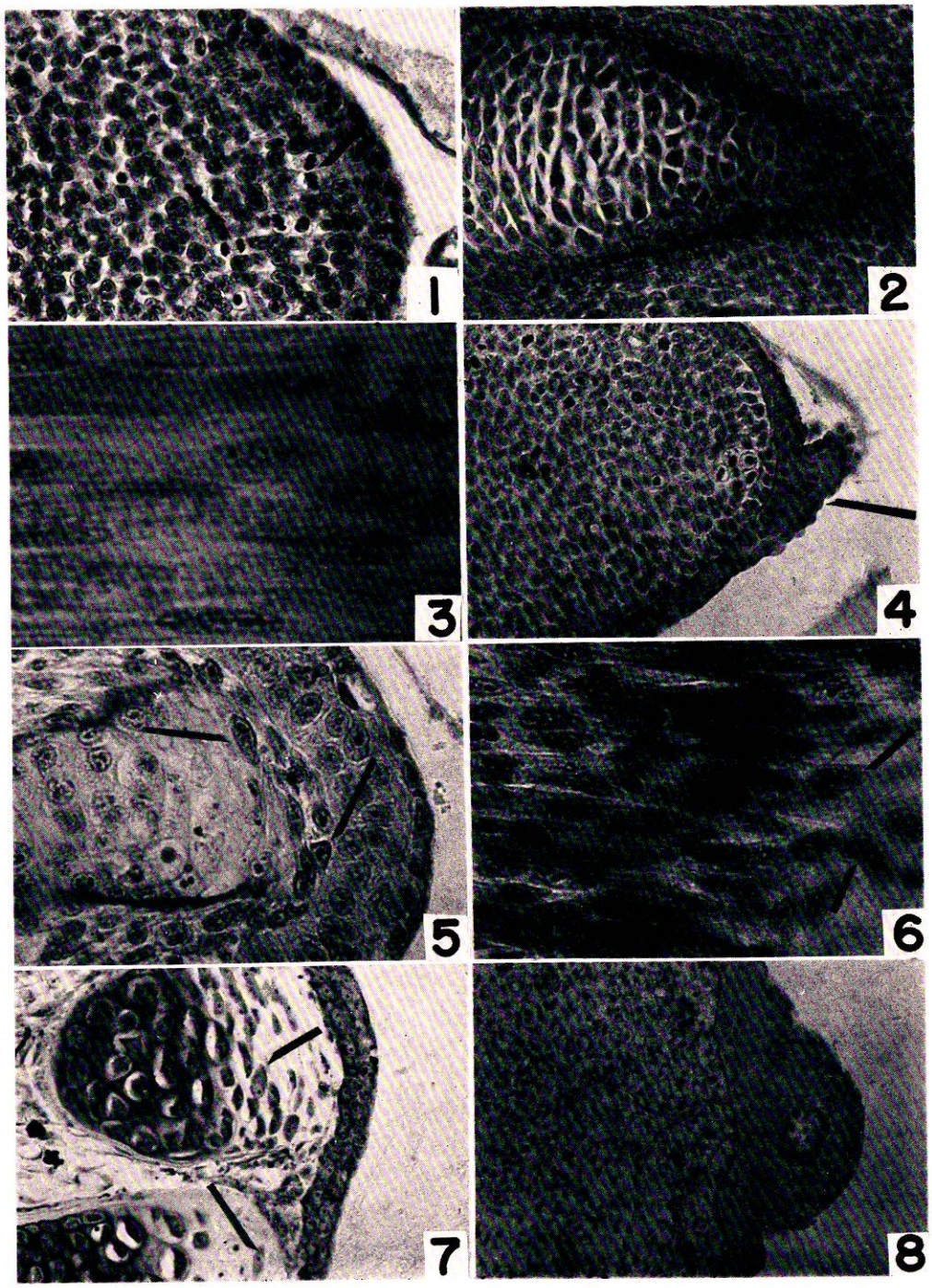
Fig. 4. A forelimb bud of a larva at stage 52 five days after amputation. Wound epidermis forms an apical cap (arrow). The cells accumulating beneath the apical cap showed no difference from mesenchymal cells. Azan. ×200.

Fig. 5. A regenerate two days after amputation of a forelimb bud of a larva at stage 55, showing blastema cells (arrows) originated from the cartilage. Azan. ×400.

Fig. 6. A regenerate of a larva at stage 57 two days after amputation, showing blastema cells (arrows) derived from muscle cells. Azan. ×400.

Fig. 7. A regenerate of the forelimb from a larva at stage 57, two days after amputation. Note a distinct nucleolus in blastema cells (arrows) of dedifferentiating cartilage. Toluidine blue. ×200.

Fig. 8. A regenerate from a larva at stage 61, showing accumulation of blastema cells of different origins beneath the apcial cap. Azan. ×100.



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