Further Studies on the Inhibitory Effects of Ca-ion on Limb Regeneration[1)]

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(With 1 Plate)

The initial process in regeneration takes place when the tissue cells that are normally surrounded by tissue fluid suddenly become exposed to an unusual *milieu interieur*, which conceivably stimulates some of the cells to dedifferentiate. Any chemical change in the culture media, or *milieu exterieur*, of the amputation surface or the regeneration blastema is, therefore, expected either to accelerate or suppress further regenerative processes. Many investigations have been conducted on the basis of this view (Rose, 1942, 1944, 1945; Thornton, 1950, 1951; Flinker and Sidman, 1953; Scheuing and Sidman, 1953; Singer *et al.*, 1955; Scheuing and Singer, 1957).

The present author has found that the isotonic calcium chloride ($CaCl_2$) solution has an inhibitory effect on forelimb regeneration in young adult *Xenopus laevis* (Nakamura, 1968). Further studies revealed that the degree of inhibitory action of Ca-ion depended upon the level in the limb at which the amputation was made. The present paper deals with the histological analysis of the action of Ca-ion on the amputated forelimbs in adult *Xenopus*.

Materials and Methods

All the toads, *Xenopus laevis*, used in these experiments were raised in the laboratory from eggs. Only young toads, about three-month old, were employed.

Amputation was performed at two different levels of the forelimbs: at the joint between the humerus and the radio-ulna (Group I), and at the middle portion of the radio-ulna (Group II). Taken out of water, animals were operated with a sharp razor and placed in isotonic $CaCl_2$ solution (0.43%) maintained at room temperature (18–22°C). Similarly operated toads were placed in tapwater and served as controls. Also a few operated

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animals were kept in Ringer solution or 0.65% NaCl solution. About 30 minutes following the operation, bleeding stopped and culture media were renewed twice within a day. The solution was changed once every day for the first two weeks and once every two or three days thereafter. The volume of culture media was 150 ml per an animal. Toads were not fed for 5 days following the operation to avoid contamination in ionic constitution of the solution. After the sixth postoperative day animals were fed twice a week.

Fixation of tissues was made in Helly's fluid for 3 hours followed by, when necessary, decalcification in Plank Rhychro's solution for 24 hours. For neutralization the material was immersed in 5% sodium sulphate solution for 24 hours. Serial paraffin sections were cut at 8μ in thickness and stained with Delafield's hematoxylin and eosin. Some sections were treated with Heidenhain's Azan stain for cartilage and other connective tissues.

Results

The regenerative process in *Xenopus* forelimbs is divided into the following three stages: the wound healing, the demolition and the blastema formation stages. Histogenesis in amputated limbs was compared between the two experimental groups at each of these three stages.

Group I (*Animals with the forelimbs amputated at the joint between the humerus and the radio-ulna*).

1) *Wound healing stage*: Immediately after the operation, the collagen layer and the mucous glands adjacent to the wound were seen retracted to some extent toward the proximal part. The wound usually closed within three days. Epidermal cells migrated toward the cut surface. The basement membrane beneath the stump epidermis was not extended toward the cut surface. A fibrous substance and the colloidal material, both stained with aniline blue, were observed beneath migrated epidermal cells.

The wound healing process proceeded in nearly the same manner in the experimental and the control groups, except that in the experimental animals many columnar cells in migrating epidermis had a large vacuole in their cytoplasm.

2) *Demolition stage*: Experimental animals remained longer in this stage than the controls. In the former, the glands and collagen layers beneath the stump epidermis were more retracted toward the proximal region than in the controls (Fig. 1). Also the colloidal material was found to be accumulated in quantities beneath the wound epidermis around the distal part of the humerus in experimental animals.

Fifteen days after the operation, the muscular bundles, by losing contour of some bundles and turning into a colloidal material containing several nuclei, took an appearance of dissolution (Fig. 2). Such a feature was never observed in the control animals.

3) *Dedifferentiation and blastema formation stage*: About 20 days after the operation, the wound epidermis began to fold and differentiation of a new

cartilage commenced around the perichondrium of the humerus' old cartilage region. There was the first sign of dedifferentiation of muscle in the experimental animals, giving rise to many dedifferentiated cells, in contrast to the control animals, in which the first sign of dedifferentiation of muscle was already evident in about 7 days after operation.

At the 40th postoperative day, the new cartilage, occupying the entire region at the level of amputation, was formed to make a thick wall which separated the stump from the poorly regenerated area consisting of a small number of blastema cells and wound epidermis (Fig. 3). That was a feature characteristically found in the calcium-treated animals only and neither the controls nor the animals treated with 0.65% NaCl solution and Ringer solution showed such a feature. The blastema cells increased in number under the wound epidermis, but the apical cap was still thick, indicating that elongation of the blastema did not occur yet. Neither glands nor muscle were seen in the blastema. In control animals at this period, the regenerate was elongated and consisted of a rod-shaped cartilage surrounded by connective tissue cells.

In experimental animals at as late as the 400th postoperative day, no indication of blastema elongation was detected and histological condition of their wound area was almost unchanged from that in the animals at the 40th postoperative day.

Group II (*Animals with the forelimbs amputated at the middle point of the radio-ulna*).

1) *Wound healing stage*: Both the experimental and the control animals completed wound healing within three days following the operation. The process was almost the same as in Group I.

2) *Demolition stage*: The demolition area was wider in range and the stage lasted longer in the experimentals than in the controls. Histogenesis proceeded almost in the same manner as in Group I.

3) *Dedifferentiation and blastema formation stage*: Seven days after operation, the experimental animals had many blastema cells derived from the periosteum of the radio-ulna (Fig. 4), but no dedifferentiation was recognized of their injured muscle. In the control animals at the same postoperative day, on the other hand, many blastema cells were found to be derived from both the radio-ulna and the muscle.

The blastema became elongated externally in 20 days after the operation, being delayed a little as compared with the controls. Differentiation of a new cartilage was commenced not only around the periosteum but in the distal portion of the radio-ulna (Fig. 5). Dedifferentiation of the muscle was evident even in wider area. Those dedifferentiated cells from the muscle migrated into the space between the wound epidermis and the distal part of the new cartilage. Both experimental and control animals had several, large multinucleated cells adjacent to the cut end and around the periosteum of the radio-ulna (Fig. 6). These cells

seemed to correspond to the osteoclasts described previously by Goode (1967) in *Bombina* and *Discoglossus*.

About 100 days following the operation, no distinct difference was found between the experimental and the control animals concerning the growth of the blastema. The new cartilage became longer, taking a spike-like shape (Fig. 7), where the cell nuclei were small and the nucleoli had disappeared. Neither epidermal glands nor muscle were seen at the distal portion of the blastema.

Discussion

In the early stage of regenerative process, injured tissues dedifferentiate and, then, their cells form the blastema. And if dedifferentiation of the tissues does not occur at the adequate time, no sequential process of regeneration will follow (Gidge and Rose, 1944; Rose, 1944). In the present study, the main injured tissues composing the amputation surface were the muscle of animals in the group I, or the muscle and bone of animals in the group II. However, in the experimental animals of either group that were placed in $CaCl_2$ solution following amputation, no dedifferentiation occured in the muscle. On the other hand, only in the group II experimental animals many dedifferentiated cells were found to be derived from the injured bone together with the osteoclasts. Therefore, it may be concluded that the blastema in the group II animals was formed by dedifferentiated cells of the injured bone and that failure of the blastema formation in the group I animals was due to absence of dedifferentiated bone cells.

The results obtained in the present study are compatible to the author's previous suggestion that the Ca-ion acts not systemically but only locally in the wound area (Nakamura, 1968). It was not determined, however, whether the long demolition stage disordered the sequential process of muscular dedifferentiation or the Ca-ion acted directly on injured muscle.

Summary

The regenerative process of the forelimbs cut at different levels was investigated in young toads, *Xenopus laevis*, which were maintained in isotonic $CaCl_2$ solution following the amputation. When the cut was made at the joint between the humerus and the radio-ulna the regenerate did not grow, whereas if the forelimbs were amputated at the middle portion of the radio-ulna the regenerate grew. Different results obtained in the two experimental groups were considered to be due to the difference in composition of the tissues at amputation surface, because in $CaCl_2$ solution injured bone dedifferentiated but the muscle was prevented from performing dedifferentiation.

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Explanation of Plate XII

Figures 1–3. Longitudinal sections through the regenerating forelimbs of the group I experimental animals.

Fig. 1. The stump 10 days after amputation, showing the retraction of the epithelial glands and thick collagen layers under the epidermis (arrow). *h*: humerus (uninjured). Azan. ×20.

Fig. 2. The stump 15 days after the operation. Note the changes in appearance of muscle. Cut level to the right. *co*: colloidal substance, *n*: nucleus. Azan. ×280.

Fig. 3. The stump, 40 days after amputation, showing no evidence of growth of the regenerate. *nc*: new cartilage, *h*: humerus. Hematoxylin and eosin. ×20.

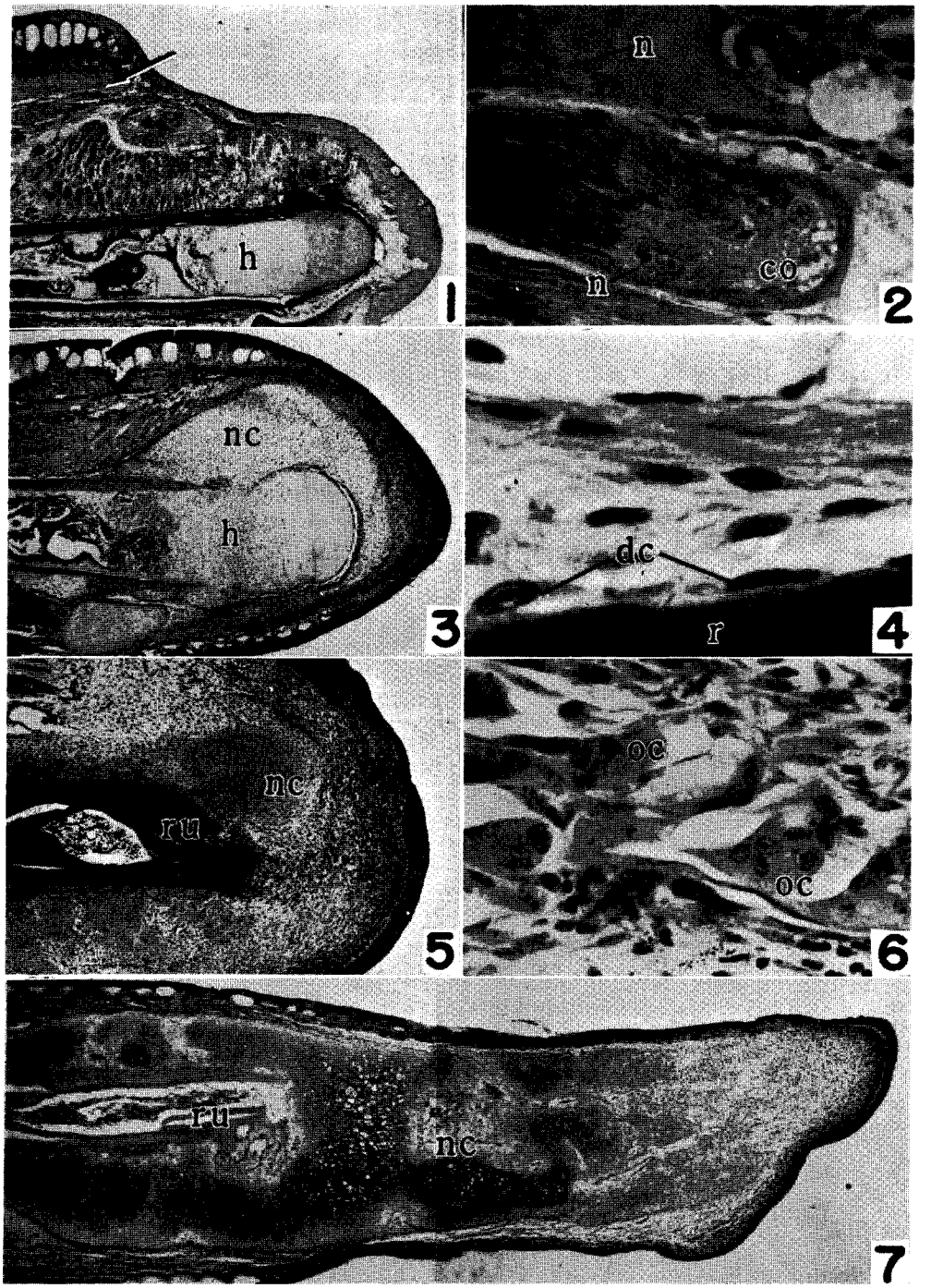
Figures 4–7. Longitudinal sections of the forelimbs of the experimental animals in the group II.

Fig. 4. A portion of the stump tissue 7 days after amputation, showing dedifferentiated cells (*dc*) derived from the radio-ulna (*ru*). Hematoxylin and eosin. ×400.

Fig. 5. The stump 20 days after amputation. A new cartilage (*nc*) is formed around the injured radio-ulna (*ru*). Hematoxylin and eosin. ×28.

Fig. 6. A portion of the stump tissue 20 days after the operation. Note the osteoclasts (*oc*) derived from the injured radio-ulna. Hematoxylin and eosin. ×200.

Fig. 7. The regenerate 100 days after amputation. A newly formed cartilage (*nc*) takes a spike-like shape. *ru*: radio-ulna. Hematoxylin and eosin. ×28.



H. Nakamura: Effects of Ca-ion on Forelimb Regeneration