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Studies on the Determination Factors in Amphibian Limb Regeneration

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Opinions are still divergent concerning the origin of cells which constitute the regeneration blastema and the factors that determine the character of the regenerated limb. Using the adult newts, *Triturus pyrrhogaster*, the senior author (1943a) previously performed several experiments on this point, by means of interchanging the tissues between fore- and hind-limbs. The results were that the determination factors locate in every tissues such as muscle, bone and skin. At that time he was much impressed with the fact that when one or more components of limbs are exchanged, whether the regeneration blastemata give rise to donor-like or host-like structures depends upon the relative amount of tissues which are comprised in a stump of the limb, more precisely speaking, upon the relative number of cells which originate from either graft or host tissues. When the number of cells derived from the host tissues is preponderant, the regeneration blastema will differentiate into a host-like structure, whereas when the number of cells coming from the graft tissues is greater, the blastema will develop as a graft-like structure.

Butler (1933) found that the irradiation of x-rays suppresses the regeneration of amphbian limbs. Therefore, when the component tissues of a limb are exchanged, subsequent to the exposure to x-ray, with the unirradiated tissues of another individual, the regeneration blastema will be formed solely or at least mainly with the cells that come from the grafted tissues, and consequently will give rise to a donor-like structure. Thornton (1942) made a similar experiment on a muscle of a young adult of *Triturus viridescens* and obtained the expected result. The present experiments are undertaken to substantiate this point with more component tissues, using or not using the x-ray as the inhibitor of regeneration from the host tissues.

Material and Methods

Adult newts, *Triturus pyrrhogaster*, were used as materials. Animals were etherized and disinfected with alcohol. The body was covered with wet gauze and fastened to an operation bed with cotton yarn. Along the dorsal side of the upper arm of the right forelimb was made an incision, through which component tissue or tissues were removed as thoroughly as possible, taking care to minimize any injury to other tissues, and then a given tissue or tissues isolated from the hindlimb of another individual were planted in substitution. The skin bordering the wound was sewn with fine thread, and the animals were kept in a wet chamber for about 20 days. Following the wound healing, the forelimb which had been operated on was amputated at the level through a distal third or fourth of the upper arm where the graft existed, with the intention of leaving the greater part of the graft within the stump. Animals thus operated on were kept again in the wet chamber for a fortnight and removed to the tap water thereafter. They were brought under observation at intervals.

In order to prevent the host tissues from regenerating, the forelimb of the host was previously subjected to x-ray irradiation. A preliminary experiment proved that in the case of our apparatus (150 Kv., 3 MA., 160 R. U./min., Dist. 20 cm.), an adequate dosage of x-ray was 1000 R. U..

Refering to the senior author's sketches (1943b), the regenerated structures were identified as the forelimb or hindlimb, i. e., number of digits, an arrangement of inner skeletons and an articulation between zeugopodium and stylopodium were taken into consideration. In some cases, however, the identification was impossible because of the super- or subregeneration of digits, irregular arrangement of inner skeletons and other malformations. These regenerates were grouped as the misbuilding in the following tables.

Experimental results

a) *Interchange of tissues between forelimb and hindlimb* This comprises three series of experiments. In the first series (A) the muscle was removed from the upper arm of the forelimb with a substitution with that of the hindlimb. In the second series (B) a humerus of the forelimb was taken out, and a femur was put in its place. In the third series (C) a humerus and the surrounding muscles were extirpated as thoroughly as possible, the nerves alone being left, and a femur together with adjacent muscles isolated from another individual was implanted into the skin sac. As control intact forelimbs of other animals were cut at the level a little proximal to the elbow. In every series of experiments as well as in controls, the regeneration blastemata were produced within 10 days after the amputation, when the animals did not fail to regenerate. They developed the processes at their distal ends as a sign of digit formation after 10 days under the laboratory conditions. In the rate of the regenerating process we could not recognize any noticiable delay in the experimental animals as compared with the control ones. After 90–120 day's rearing, the regenerates were all sketched and fixed to examine their inner structures. The results are shown in Table 1.

Table 1. Interchange of various tissues between fore- and hindlimbs.

Series	No. of operation	No. of death	No. of non-reg.	No. of available cases	Results			
					Hind limb	Fore limb	Duplicate limb	Mis-building
A	93	28	2	63	20	34	6[1)]	3
B	47	10	1	36	4	29	1[2)]	2
C	75	30	3	42	11	18	7[3)]	6

1) Out of 6, 3 are duplicate limbs of 5 and 4 digits each, one is of 5 and 2 digits and the remaining 2 are of 4 and 3 digits.

2) It is a duplicate limb of 4 and 3 digits.

3) Out of 7, 3 are of 5 and 4 digits and one is of 5 and 2 digits. The remaining 3 are of 4 and 4, 4 and 2, 4 and 1 digits respectively.

In view of the injury of tissues by the implanting procedure as well as the lessening of their vitality after planting, the high proportion of forelimb-fromation is not impossible to understand. However, as noticed in the table, the hindlimbs are also produced in every series. This means that both muscular and bony tissues of the hindlimb are enough to be endowed with a morphogenetic factor which characterizes the regenerate to become the hindlimb, although it seems different in effectiveness between the two tissues. Among three series, the results of series C are surprising but suggestive, because the forelimbs are produced more often than the hindlimbs in spite of the host's skin and nerves alone being left. The nature of the nervous influence is now obscure, but it is generally accepted to be essential to the inception of regeneration, its morphogenetic rôle being not yet demonstrated. The predominant production of the forelimb in this case, therefore, may be attributable to the skin presented at the wound surface. In other words, it may be inferred that the skin, presumably its hypodermis, is also endowed with a morphogenetic factor, although the interchange of skin alone between the fore- and hindlimbs yielded negative results.

b) *Substitution of hindlimb-tissues for x-rayed forelimb-tissues*

Newts were anesthetized with ether and shielded with leaded rubber sheet except for the right forelimbs, which were exposed to an x-ray dosage of 1000

R. U.. One day after the exposure, the following four series of experiments were carried out: series A to series C were comparable each with series A to series C of the preceding experiments, except that the forelimbs of hosts were irradiated. In series D the humeri were removed together with their surrounding muscles from the irradiated forelimbs, and were replaced by the muscles of thighs of unirradiated animals. In every series the specimens operated on were kept at 25 ° C in the thermostat because of the change in the room temperature. Seventy to ninety days following the amputation, the regenerates were examined as before. The results are arranged in Table 2.

Table 2. Interchange of tissues between irradiated forelimb and unirradiated hindlimb.

Series	No. of operation	No. of death	No. of non-reg.	No. of available cases	Results			
					Hind limb	Fore limb	Duplicate limb	Mis-building
A	30	3	11	16	3	3	0	10
B	30	1	7	22	3	3	2[1)]	14
C	31	7	3	21	6	0	3[2)]	12
D	20	4	7	9	2	0	0	7

1) One is a duplicate limb of 5 and 4 digits and the other is of 5 and 2 digits.
2) One is a duplicate limb of 5 and 2 digits and the other two are of 4 and 3 digits.

As shown in Table 2, the regeneration was suppressed completely as much as 29 per cent in all viable cases. This may be caused, as Lichko (1934) pointed out, by the quick development of fibrous scar tissue at the amputation surface in the case of x-ray irradiation. The same reason would also account for a higher percentage of the occurrence of misbuilding in this case than in the preceding experiments. At any rate, it can be stated that in every series the hindlimbs occurred more often than in the preceding experiments. The result is as expected. Out of five duplicates, three limbs that possess 5 digits are probably hindlimbs.

Discussion

As to the origin of the cells which form the regeneration blastema of amphibian limbs, various opinions have been expressed by the previous investigators. An old concept that the regeneration blastema arises from as many sources as the component tissues which exist at the level of amputation, and that each tissue can provide only the cells of its own kind, was thoroughly disproved by Weiss' excellent experiment (1925) which demonstrated that the blastema produced at the end of a boneless stump was able to develop a new distal part of the limb, equipped with its typical skeleton. He seems of the opinion that a large portion of the blastema is made up by indifferent reserve cells in the various connective tissues of the stump, and a little, supplemented by differentiated cells immigrating from the muscle and bone in the stump (1939). Toyomasu (1938) argues also the connective tissue origin of the blastema. On the other hand, Hellmich (1931) claims that the blastema comprises both of histogenic and haematogenic cells. Further, Thornton (1938) and others believe that the component tissues in the stump dedifferentiate and contribute cells to the blastema.

From the results of the present experiment, it seems safe to state that each grafted tissue of limb such as muscle and bone as well as host's skin can supply the cells for the blastema formation, provided that the faculty of dedifferentiation and proliferation differs in different kind of tissues. Especially, in the case where the unirradiated hindlimb tissues were substituted for the irradiated forelimb ones, the majority of cells forming the blastema were surmised to arise from the grafted tissues. But it cannot be said that the implanted tissues alone were the source of these cells, although the x-ray dosage was strong enough to suppress the regeneration from the irradiated control limbs. The reason for this is that we cannot refute the possibility of the irradiated tissues being induced to recover the faculty of producing the blastema by the implanted active tissues. The skin, at least its epidermis, of the regenerate should be derived from the irradiated skin of the host in this way. The production of the forelimb in the present x-rayed experiment would be understandable also to be such a case.

At all events, our present problem concerns the occurrence of the hindlimb in the case where the hindlimb tissues were implanted into the forelimb, from which tissues had been removed previously. It must be stated here, as pointed out by Thornton (1942), that the implanted muscle or bone consisted not only of muscle or bone proper, but also of some connective tissue sheath. There is a possibility that this connective tissue takes part in the blastema formation. But it seems improbable that this tissue alone is a source of the blastema cells, although we lack good evidence against it, as far as the present experiments are concerned. As stated above, we are of the opinion that all the component tissues, including the connective tissue, contribute cells to the regenerating blastema.

Summary

1) Hindlimb muscle or/and bone of adult *Triturus pyrrhogaster*, implanted into the forelimb from which muscle or/and bone had been previously removed, was/were able to regenerate the hindlimbs in about 30 per cent of the discriminative cases.

2) When unirradiated hindlimb muscle or/and bone was/were implanted into the irradiated forelimb, the production of the hindlimbs became as high as 70 per cent of the distinguishable cases.

3) It is clear from the above data that the regenerating blastemata are derived mainly from the implanted tissues in the latter experiment.

4) It is evident from the present experiments that the component tissues of limb are each in possession of factors which determine the pattern of development of the blastema.

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