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EXPERIMENTS ON THE DEVELOPMENT OF THE LIMBS IN AMPHIBIA

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Although previous experiments by Barfurth,[1] Byrnes,[2] Kammerer,[3] Braus,[4] Lewis[5] and others have yielded many interesting facts regarding the development and regeneration of the limbs in Amphibia, the bounds of the limb rudiment at very early stages have not been determined exactly in any species, and it is not known how wounds of a definite size and character in the limb region affect the subsequent power to give rise to a normal appendage. In order to control properly certain transplantation experiments upon the fore limbs in *Amblystoma*, relating chiefly to questions of position and laterality, it was, therefore, found necessary to determine first the regenerative power of the tissues of the limb region after the removal of the limb rudiment in whole or in part before differentiation had begun. Accordingly the following experiments were made:

1. Simple extirpation of the body wall of the fore limb region at a period before the limb bud becomes visible on the surface, the size of the wound being varied.

2. Extirpation of a definite portion of the limb region.

3. Removal of the limb rudiment and subsequent covering of wound with ectoderm taken from some other region of the embryo.

4. Removal of the mesoderm alone from the limb region, afterward replacing the overlying ectoderm.

5. Removal of the ectoderm alone leaving the underlying mesoderm intact.

6. Transplantation of small masses of mesoderm, not yet visibly differentiated, from the limb region to pockets under the skin on the side of the embryo.

The fore limb appears at a much earlier stage than the hind limb in the Urodeles and much earlier than either extremity in the Anurans. The operations were consequently done upon embryos about the time

Table showing effect of removal of body wall of fore-limb region, the number of cases in each category being given

SIZE OF WOUND	WOUND NOT COVERED				WOUND COVERED			
	Not cleaned		Cleaned		Not cleaned		Cleaned	
	Regen.	Not regen.	Regen.	Not regen.	Regen.	Not regen.	Regen.	Not regen.
Not recorded	11	9			2	8		
3 somites	22	1	13	12			7	14
*3 somites							3	2
3½ somites			1	0			1	0
*3½ somites							0	1
3½ somites	25	8	2	7			0	11
*3½ somites							0	2
4 somites	16	2	2	3			0	3
*4 somites							0	2
4½ somites	2	0						
Total	76**	20	18	22	2	8	11	35
Percentage	79	21	45	55	20	80	24	76

* In the groups so marked the ectoderm of the limb region was healed back in place; in the other groups in which the wound was covered ectoderm from other regions of the body was used.

** Includes five cases, constituting the fifth group of experiments, in which a large part of the mesoderm was purposely left in.

of appearance of the tail bud (fig. 1). In many cases, however, the embryos were slightly younger or slightly older than the one shown in the figure. The fore limb of *Amblystoma* develops as a thickening of the somatopleure which centers in the region of the fourth myotome and extends over into that of the third and fifth. In order to remove the tissues which form the appendage, a circular incision of about the diameter of these three segments was made with fine scissors, and the loosened piece including both ectoderm and mesoderm was then torn free and removed. The few mesoderm cells which are usually left sticking to the pronephros and around the edges of the wound were carefully removed in some cases and in others left in place. In some individuals the pronephros was left intact and in others it was removed. In nearly all cases the operation was done on one side, the other being left in its normal condition for comparison.

Experiments. 1. The results of the simple removal of the body wall of the limb region are given in the left half of the Table. While the figures in the single classes are often not large enough to give significant percentages, it is clear that the cleaning of the wound of all scattered mesoderm cells reduces very materially the proportion of cases in which regeneration occurs, i.e., from 79 to 45 per cent. It is also apparent that when the size of the wound exceeds in diameter the length of three somites the proportion of cases in which limbs develop is considerably less, whether the wound is cleaned or not. When it is carefully cleaned and exceeds three somites in diameter, the healing is slow and there is a very high mortality, which accounts for the small number of experiments with wounds of this size appearing in the tabulation.

The age of the embryos, within the limits of the stages experimented upon, has no influence upon the regenerative capacity. The presence of the pronephros, which on account of its close proximity might possibly

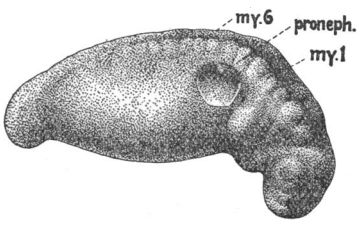

FIG. 1.

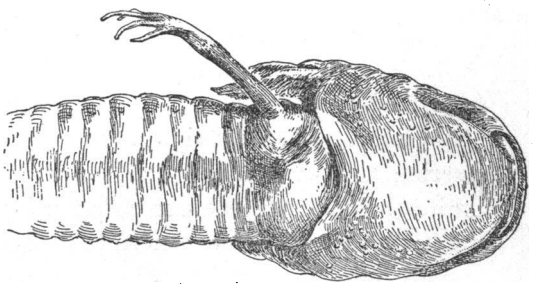

FIG. 2.

FIG. 1. EMBRYO OF AMBLYSTOMA PUNCTATUM SHOWING THE WOUND IMMEDIATELY AFTER OPERATION IN A TYPICAL EXPERIMENT. × 10.

FIG. 2. AMBLYSTOMA LARVA FROM WHICH THE LEFT FORE LIMB RUDIMENT WAS REMOVED, PRESERVED 87 DAYS AFTER OPERATION. × 5.

be thought to have some influence on the limb, seems not to affect its normal development, for a perfectly normal limb may be formed when the pronephros is removed. The removal of this organ facilitates, however, the cleaning away of the mesoderm cells and thus may affect indirectly the results of experiments, even though it exerts no formative stimulus.

The limbs which develop after removal of the rudiment are retarded in development to a varying degree. In no case has a limb been found subsequently to arise, when the negative condition has persisted till the twelfth day, and in only three cases when it has remained till the tenth. Consequently, if development has not begun by the end of the second week it is safe to assume that it will not take place at all. A large number of cases observed till the end of the third or the fourth week, and in some instances much longer, show this to be true (fig. 2).

No extensive examination of the material has been made by means of sections, but five cases which were cut show, that even though the free appendage is lacking, a shoulder girdle of somewhat reduced dimensions is developed on the operated side, just as Braus[6] has found in the case of *Bombinator*.

2. The experiments of the second group, in which limited portions of the limb rudiment were removed—dorsal, ventral, caudal, or rostral half, or the central portion—all resulted in the formation of perfect limbs whose development was but slightly retarded.

3. A comparison of the right and left halves of the Table shows at a glance that the covering of the wound with indifferent skin is a considerable hindrance to the development of the limb. The cases, cited on the first line of the table, in which the wounds were not cleaned happen to give, however, a considerably exaggerated idea of the effect of skin transplantation, since the control experiments in which the wound was left uncovered, made at the same time and with the same material, showed also a very high percentage of cases of non-development. Some unknown factor must have affected the results here. The cases in which the wound was cleaned are gathered, however, from experiments made at various times, and these show very clearly the effect of covering the wound. In the experiments with wounds of but three somites in diameter the percentage of development is reduced from 52 to 33, while in the cases where the wound is three and a half segments or more in diameter, i.e., when the region of the anterior half of the sixth segment is included, the percentage of development is reduced to zero. The determination of this latter point is of prime importance for the main purpose of this work, since it shows how much it is necessary to remove, when transplanting other buds to the limb region, in order to be sure that the appendage which develops is not simply regenerated from the host.

4. The experiments in which the ectoderm of the limb region was healed back in place after removal of the mesoderm are only ten in number and are, therefore, insufficient to give significant percentages. They nevertheless confirm the result of the previous series. Five experiments with wounds of three segments in diameter yielded three cases in which development occurred and two in which it did not occur. Five other experiments with wounds four segments in diameter were all negative. The mesoderm was carefully cleaned off at the time of the operation in all experiments of this set.

5. The five cases in which the ectoderm alone was removed and the mesoderm, at least in great part, left in place all resulted in the develop-

ment of a normal limb. Since these differ only in degree from those experiments of the first group in which the wound was not carefully cleaned, they have been included in the tabulation under that class.

6. To transplant the mesoderm alone, the ectoderm is first torn free from the underlying tissues after the usual circular incision is made. The mesoderm of the region is then cut out in a single piece as large as possible, and transferred with a blunt needle to a small pocket under the ectoderm of another embryo previously prepared. The mass of cells is then pushed into the pocket and left, a procedure that is not always easily carried out because of the extreme stickiness of these cells.

Nineteen experiments were made, in four of which the embryo died before yielding results. In five cases the transplanted tissue was entirely resorbed; in three others it remained a small nodule; in one a long appendage without digits resulted; and in six there developed a limb of approximately full size which, however, in most cases showed some irregularities, usually in the form of reduplications.

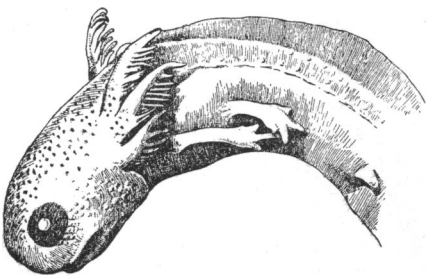

FIG. 3. AMBLYSTOMA LARVA WITH SUPERNUMERARY FORE LIMB, DEVELOPED FROM CELLS INOCULATED FROM THE MESODERM OF THE LIMB REGION OF AN EMBRYO IN THE STAGE SHOWN IN FIGURE 1. EXPERIMENT TR. MES. 1. PRESERVED 16 DAYS AFTER OPERATION. × 10.

The most perfect of these showed reduplication of only a single digit; another gave rise to a perfect limb with complete reduplication of fore-arm and manus. The one shown in figure 3 has the beginning of a supernumerary digit on the radial side of the hand. The other cases were more irregular. Sections show that in addition to the free appendage a small shoulder girdle is formed from the transplanted tissue. This is also the case when the entire limb rudiment is transplanted, as is shown both by Braus's[7] experiments upon *Bombinator* and my own upon *Amblystoma*.

Conclusions. The anterior limb of *Amblystoma*, according to the foregoing experiments, is already determined, at the time of appearance of the tail bud, in the mesoderm cells of that region of the body wall which lies close to the pronephros and ventral to the third, fourth, and fifth myotomes. The prospective significance of this group of cells as a whole thus is defined some time before differentiation becomes visible. Forming a narrow zone around this region there are cells which may vicariously give rise to a limb in case the rudiment proper is removed. These cells, responding to the stimulus of the wound, gradually move up toward the center, and covering the bare yolk, afterward in many

cases develop into a normal limb. Miss Byrnes[8] some years ago called attention to this phenomenon in the case of the hind limb rudiment of *Rana* embryos, though the results of the present study, which has aimed to determine exactly the limits of the power to regenerate, show that it is much more restricted than she supposed to be the case. Perhaps, however, there is in *Amblystoma* the same difference in regenerative capacity between fore and hind limb regions that Braus,[9] found to be the case in the Anurans. Covering the wound with indifferent ectoderm, or healing back the original ectoderm of the limb region, hinders this movement of peripheral cells toward the nodal point from which the new limb may arise, and definitely prevents development, provided that the extent of the wound includes the region of the anterior half of the sixth somite in addition to that of the third, fourth and fifth. In case the wound is but three segments in diameter, development may or may not be prevented but the proportion of negative cases is considerably larger than when the wound of the same size is left uncovered.

Carried out upon a different form and on much younger embryos than the experiments of Braus, the present experiments afford additional evidence that the mesoderm cells of the limb region, while forming a definitely differentiated system as a whole, are nevertheless totipotent within that system as far as the skeletal and muscular elements of the limb are concerned. They can give rise to a perfect limb when placed in an unusual environment, even when the original arrangement of the cells is disturbed, as it necessarily is when the mesoderm alone is transplanted; and a small part can develop into a whole. Other experiments, not described here, show, however, that at the time of operation differentiation has already begun to some extent, though it has evidently not become irreversible. The ectoderm merely serves as a covering to the limb and no indication of any specific stimulus from this layer could be detected. Other structures in the region such as the myotomes, as shown by Byrnes (1898) and Lewis (*op. cit.*) or the pronephros, as found in the present investigation, do not affect the development or regeneration of the limb bud in any way.

[1] D. Barfurth, *Arch. EntwMech., Leipzig*, 1 (1894).

[2] E. F. Byrnes, *J. Morph., Boston*, 14 (1898); *Anat. Anz., Jena*, 15 (1898); *Arch. EntwMech.*, 18 (1904).

[3] P. Kammerer, *Arch. EntwMech.*, 19 (1905).

[4] H. Braus, *Münchener Med. Wochenschr.* (1905); *Morph. Jahrb., Leipzig* 35 (1906).

[5] W. H. Lewis, *Anat. Record*, 4 (1910).

[6] H. Braus, *Morph. Jahrb.*, 39, 194 (1908).

[7] H. Braus, Ibid., p. 317.

[8] E. F. Byrnes, *Anat. Anz.*, 15 (1898).

[9] H. Braus, *Morph. Jahrb.*, 35 (1906).