Surface potentials and the control of amphibian limb regeneration

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SUMMARY

The relationship between surface potentials and amphibian limb regeneration is examined. The wound surface becomes increasingly positive for several days after amputation but then decreases again, often becoming negative for a variable period during blastemal growth. The same changes of surface potentials are observed during wound healing alone, in the absence of amputation and following amputation of irradiated or denervated limbs. Similar changes occur in non-regenerating frog arms. These observations and other cited reasons lead to the conclusion that surface potentials do not control regeneration.

INTRODUCTION

It has been clearly demonstrated that the appearance of a regenerative morphogenesis in amputated amphibian limbs is conditioned by the presence of nerves (Singer, 1954) but the mechanism by which the nervous system induces such a morphogenesis is, on the other hand, as yet little understood. Amongst the different explanations which have been put forward, there is one which proposes surface bioelectricity as the regulating factor in morphogenetic processes (Becker, 1961). According to this theory, potential differences which can be found on the external surface of the tegument of Amphibians, along the proximodistal axis of the limbs, derive from the nervous system (Becker, 1961) and in particular from Schwann’s sheath surrounding the nerve fibres of the brachial nerves (Becker, 1974), and these surface potentials (SP) constitute the mechanism for the nervous control of regeneration. This interpretation depends fundamentally on two types of experiment by which it has been possible to establish a relationship between SP and regeneration. The first experiment involves section of the brachial nerve, which brings about the disappearance of the limb’s capacity for regeneration and the abolition of SP (Becker, 1961). The second procedure shows notable differences in the evolution of SP during the regeneration of the limb of a triton (Urodela) and the healing of the limb of a frog (a species which does not regenerate in its adult state) (Becker, 1961). In fact, section of the limb of a urodeles amphibian instigates an initial inversion of polarity leading to the

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appearance of a strong positivity. This declines rapidly, and, from the sixth day following amputation the stump becomes strongly negative for about 10 days. Finally, the initial polarity gradually re-establishes itself. In the Anura, although section of the limb is similarly followed by a strong positive distal charge, there is, by contrast, no subsequent negative phase, and the SP gradually regain their initial value as healing proceeds. According to Becker, the negative phase which can be seen only in the urodeles corresponds to the differentiation and growth of the blastema.

This theory, which has frequently been taken up and cited by authors studying regeneration (Smith, 1967; Rose & Rose, 1974), provides an attractive explanation of the function of the nervous system in regeneration. Now, when we undertook the study of SP in Pleurodeles limbs (Lassalle, 1974a), we demonstrated that, contrary to the observations reported by Becker, SP do not originate in the nervous system but in the epidermis (Lassalle, 1974b) and that they derive from a difference in the permeability of the tight-junctions along the proximodistal axis of the limb (Lassalle, 1975, 1976) but not from difference of Na-K pump activity (Lassalle, unpublished results). This difference in permeability (the most positive area being the most permeable one) is translated into bioelectrical terms through the existence of unequal values of transepithelial potentials (observable between the internal and external areas of the skin) in the proximal and distal regions of the limb, the most permeable area having the smallest difference in transepithelial potential (Lassalle, 1976, 1977). Given the origin of SP, which has since been confirmed by some authors with regard to Triturus viridescens (Borgens, Vanable & Jaffe, 1977) and to Rana esculenta (Fontas & Mambrini, 1977a, b), the function of SP in regeneration had to be reconsidered. We present here the results of our research conducted particularly with Pleurodeles.

METHODS AND MATERIALS

The triton, Pleurodeles waltlii Michah, and the frogs, Rana esculenta L., came from stock raised in the laboratory. All work was done at 20 ± 1 °C.

Regeneration was studied in adult Pleurodeles whose limbs were sectioned in the middle of the forearm.

Control experiments comprised measuring the SP of animals healing a wound. Two groups of animals were analysed. The first group was made up of Pleurodeles which had a piece of skin excised from a random area of the dorsal region of the limb. The second group was made up of Pleurodeles in which limb regeneration had been inhibited either by X-irradiation (2000 R) (Lheureux, 1975) or by denervation. The irradiated limbs were sectioned 15 days after irradiation; the denervated limbs, in which the circulation had been left intact to avoid regression (Revardel & Chapron, 1975), were sectioned immediately after the operation.
In some cases, measurements were also carried out on the amputated stumps of species (*Rana esculenta*) which have naturally lost the capacity for regeneration.

In all these groups, the SP were measured every 2 or 3 days in animals anaesthetized in MS 222 (1:1000). The recording electrode was placed on the wounded area, the reference electrode being located in a proximal region about 4 mm from the first. Before amputation or the removal of a piece of skin, the electrical polarity of the region of the limb which was to be operated on was recorded.

The SP were recorded with two electrodes (one as a reference, one to record) of silver-silver chloride. Contact between the animal's skin and the electrodes was ensured by a solution of NaCl at a concentration of 7 per 1000. The differences in potential were measured on an oscilloscope via a cathode follower.

**Histology**

Light microscopy: The limbs and the regenerates were fixed in Bouin's solution with or without decalcifying treatment according to the age of the particular animal. The sections were stained with azan.

Electron microscopy: The pieces of skin were fixed in 2-5 % glutaraldehyde in a 0-6 M solution of sodium cacodylate buffer (pH 7-4). After being washed in the buffer, the pieces were post-fixed for an hour in 1 % osmium tetroxide in the same buffer. Specimens were embedded in Araldite after dehydration in acetone. Semi-thin sections were stained with azure blue. Ultra-thin sections were stained with uranyl acetate and lead salts.

In order to make the extracellular permeability of the healing epidermis visible, lanthanum salts in ionic form (1-20 mM/l) were added to the physiological solution (Erlij & Martinez-Palomo, 1972). After an hour of contact, the skin was fixed using the techniques for electron microscopy.

**RESULTS**

1. *Regenerating limbs*

The 12 measurements of SP carried out on *Pleurodeles* (Fig. 1) show that amputation is followed by the appearance of a strong positivity, the maximum intensity of which ($\bar{x} = +18$ mV) is usually reached 4-6 days after the operation. This positivity decreases rapidly and towards the 15th day the polarity of the stump is reversed ($\bar{x} - 4$ mV) for about 2 weeks. Finally, around the 13th day following the amputation, the polarity of the extremity of the regenerating limb reaches a value close to that measured before amputation ($t = 0$). Figure 1 also shows that the standard errors set up at each mean are relatively small before amputation and after the 13th day, but are on the other hand of great importance between these two periods. The causes of this variability become
clearly apparent once the growth curves of the SP are analysed individually (Fig. 2). It appears that the magnitude and duration of positive and negative phases are highly variable from one limb to another; thus the positive phase, whose maximum magnitude varies between +12 mV (case no. 2) and +28 mV (case no. 1), can be reached in 3 (case no. 3) to 6 days (case nos. 1, 2, 4) and its total duration varies between 8 (case no. 2) and 6 days (case nos. 1, 3, 4). In the same way, the negative phase is sometimes of greater magnitude (−18 mV for case no. 2), but equally is sometimes very weak (case no. 3) and even non-existent (case no. 4), its duration varying between 12 (case no. 3) and 21 days (case no. 2). No relation can be established between the magnitude of these various phases and speed of regeneration.

Parallel study of histological changes reveals that healing sets in rapidly; 8 h after amputation, epidermal migration is already far advanced (Fig. 3a) and 24 h later, the wound is covered with several layers of cells. The epidermis presents at that time an undifferentiated appearance (Fig. 3b). Differentiation reappears extremely rapidly; 3–4 days later, the stratum corneum is beginning to be re-established (Fig. 3c) but at this stage the epidermis shows a high level of passive permeability (visible through use of lanthanum; Fig. 3d). Beneath the epidermis, the first signs of tissue degeneration appear: the bone is considerably eroded, the soft tissues are invaded by blood vessels and small tongues of epidermis, these last contributing to the phagocytosis of the damaged cells (Singer & Salpeter, 1961). Ten to fourteen days after amputation the phase of
tissue degeneration is over, and the distal regions of the sectioned tissues become dedifferentiated. The mesenchymatous cells resulting from these dedifferentiations accumulate beneath the epidermis and give rise, about 3 weeks after amputation, to a mass of homogeneous cells which make up the blastema. This last grows in volume and in length up to about the 40th day and then begins to differentiate.

2. Limbs healing a wound

Figure 4, giving three examples of variations in SP during the regeneration of a piece of skin of *Pleurodeles*, shows that the evolution of the SP is identical with that observed on the extremity of a regenerating limb. The initial phase lasts around 10 days, then the negative phase, of variable magnitude depending on the individual (weak in case no. 2, higher in case no. 3), lasts for some time between 8 (case no. 1) and 18 days (case no. 3). After from 15 to 25 days, the initial polarity of the wound area is re-established.
Fig. 3. For legend see facing page.
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Fig. 4. Surface potentials during the healing process following the removal of a strip of skin in three Pleurodeles.

Pleurodeles limbs whose regenerative capacity has been inhibited either by X-rays or by section of the brachial nerves show the same evolution of SP (Fig. 5a, b) as that described above for normal amputated limbs. On a histological level, healing corresponds to the closing of the wound, to the phagocytosis of damaged tissues and to the erosion of the distal region of the stump tissues.

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FIGURE 3

Wound healing in Pleurodeles.
(a) Eight hours after operation. Epidermal migration is already far advanced. The epidermal cells move from the stump to the wound (arrow). Vertical and oblique bars show the limit between the stump (S) and the regenerate.
(b) Twenty-four hours after operation. The wound is covered with several layers of cells. Wound epidermis (W.Ep.) is lying on the muscles (M).
(c) Four days after operation. The stratum corneum (SC) has reappeared.
(d) Four days after operation. The addition of lanthanum to the outside solution shows that at this stage the epidermis has a high level of passive permeability. Lanthanum deposits are visible (arrows) in intercellular spaces (IS).
In the frog, just as in the triton, limb amputation is followed by the appearance of a positive phase (Fig. 5c) whose maximum magnitude, reached towards the 15th day, is nonetheless of greater magnitude (+60 to +70 mV) than that observed in the Pleurodeles (+12 to +30 mV). The positivity decreases extremely rapidly and 10 days after amputation the SP, whilst undergoing some fluctuations in magnitude, gradually regain their initial value up to the complete healing of the limb. The negative phase was not observed in the animals we used.
DISCUSSION

Our results show that:

(1) Regeneration of the distal extremity of normal limbs of *Pleurodeles* is accompanied by a change in SP. The evolution of these last is characterized by the successive appearance of two phases of equivalent duration but opposite polarity. The first phase which is positive corresponds on a histological level to the closing of the wound, to the phagocytosis of the damaged tissues and to the beginning of cell differentiation. Its presence was observed in all the cases studied. The second phase which is negative corresponds to the end of dedifferentiation, to the building up and growth of the blastema. Its presence is not regular.

(2) The regeneration of the control animals, which is in fact restricted to the healing of a wound (closing of the wound, phagocytosis of damaged tissues and the beginning of dedifferentiation), shows an identical evolution of SP.

These last results allow us to assert that the changes in the SP observed in regenerating limbs are entirely attributable to the processes of epidermic repair following amputation and not to the mechanisms which lead to the building up and growth of a regenerative blastema, as has been suggested (Becker, 1961). Given the results which we have obtained on the origin of SP (Lassalle, 1976, 1977), it is relatively easy to establish a relation between the evolution of SP during regeneration and the physiological state of the epidermis. The increase of positivity which appears immediately after amputation corresponds to a simultaneous recording of two types of potential: firstly, the transepithelial potentials existing between the external area (negative) and the internal area (positive) of the skin, this last being made accessible to the recording electrode by the internal flow of liquids and secondly, the injury potentials, which appear between the healthy area and the wounded area, the wounded area being negative in relation to the healthy area. In these conditions, the electrode placed on the front of the section records the resultant of these potentials of opposite polarities.

A few days later (usually the sixth day), when the healing processes have advanced, the injury potentials no longer arise: since the epidermis of the healing area is still highly permeable at this stage, only the transepithelial potential (positive) is recorded. In this condition, the SP reaches maximum positivity. The epidermis progressively reassumes a normal structure, and isolates the internal area from the external area; the passive permeability and, in consequence, the positivity diminishes, and it can be supposed that, for a few days, the permeability becomes less than that of the skin surrounding the wound, whilst the SP of the regenerating zone are negative. Finally, the initial permeability is re-established once healing is completed. These variations of SP values are entirely attributable to variations of passive permeability since we showed that Na-K pump activity of the new wound epithelium is no different from that of the normal adult epidermis (Lassalle, unpublished data).
The comparison of our results with those described in the literature on the subject on the whole show numerous similarities. The initial positive phase in particular has been noted by all the authors; it is only its interpretation which varies from one author to another. In fact, Becker (1961) attributes the positivity which follows amputation to a cessation in the flow of electrons in the brachial nerves, whilst excluding the effect of injury potentials; Monroy (1941) offers an interpretation closer to our own since, for this author, the positive phase corresponds to the injury potential caused by the amputation. Very recently, Borgens et al. (1977) have put forward an interpretation which is in all points identical with our own. As for the negative phase; the inconsistency with which it occurs in recordings made with the frog and with some of the Pleurodeles explains why it has been noted extremely irregularly by the different authors and why no attempt has been made to explain it; thus Monroy does not describe it at all, Borgens et al. (1977) note it as episodic, Becker (1961) describes it only in relation to tritons; only Rose & Rose (1974) note its regular existence both amongst regenerating tritons and amongst those in whom regeneration has been inhibited by X-rays or denervation. The negative phase, to which Becker (1961) had attributed the growth and differentiation of the blastema, therefore appears highly irregular and independent of the presence of a blastema and in no way indispensable to the process of regeneration. Our argument as to the total absence of any stimulating effect of SP on regeneration is moreover reinforced by the fact that the absence of SP in Amphibian larva such as the axolotl and Pleurodeles (Lassalle & Boilly, 1977) and the frog (according to research done by Taylor & Barker, 1965) is in no way prejudicial to the establishment of the morphogenetic processes leading to the harmonious regeneration of a limb. This observation strongly suggests that the SP play no part in limb regeneration, especially since the appearance of these potentials at the time of metamorphosis (Lassalle & Boilly, 1977) is accompanied in the Anura by the loss of the capacity to regenerate and in the urodeles by a diminution in the speed of regeneration.

REFERENCES

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