Cellular events in denervated limb stumps of *Ambystoma* larvae during re-innervation and subsequent regeneration[1]

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Summary. Amputated, denervated *Ambystoma* forelimbs undergo cellular dedifferentiation and slight resorption through 12 days post-amputation. Subsequently, as limb stumps become re-innervated, there occur mitosis, blastema formation, and regeneration. The initial increase in the mitotic index in once-denervated limb stumps is closely correlated with the presence of ingrowing nerve fibres.

When amputated limbs of larval *Ambystoma* are denervated, blastemas do not develop and regeneration fails to occur[2,3]. However, dedifferentiation of stump tissue cells occurs in these denervated limbs and cells resembling blastema cells can be observed in their distal regions while the limb tissues are slowly resorbed[2,3]. If nerves are allowed to re-innervate the limb, the resorption process stops, typical blastemas develop, and ultimately regeneration is completed[2,3].

Based on these early studies and our more recent results[4], we have proposed separate roles for injury, nerves, and wound epidermis in regeneration[5]. Injury causes G_0 stump cells to dedifferentiate and progress through the G_1 and S phases of the cell cycle. Nerves are important to one or more G_2 events and the wound epidermis keeps the daughter cells progressing through additional cycles[5]. More recently it has been suggested that nerve axons may actually have to make close contacts with dedifferentiated cells[6] to impart a neurotrophic effect[7].

The early observations of Butler and Schotté[2] and Thornton[3] suggested an experimental approach to test the nerve-blastema cell contact view[6] and the proposed G_2 role of nerves[5]. Here we report the 1st results of these experiments which involved examining cellular events in denervated limbs upon re-innervation and subsequent regeneration.

Materials and methods. *Ambystoma maculatum* larvae were raised individually from eggs collected in southern Ohio and grown to a snout-tail tip length of 4.0 ± 0.5 cm. After anesthetization in MS 222, both forelimbs were amputated through the distal third of the radius and ulna. The 3rd, 4th, and 5th brachial nerves of the left limbs were denervated at the brachial plexus 1 day post-amputation. Right limbs served as controls. Beginning on day 2, 2 larvae were fixed in toto in Carnoy's fixative on each day through 14 days post-amputation and every other day thereafter. After 24 h fixation, limbs were histologically prepared in paraffin, and stained by the Feulgen method for mitotic figures. Fast green was the counter stain. Mitotic indices were obtained by examining dedifferentiated cell nuclei at 430× in grid areas (0.04 mm^2) in the distal portions of the limbs. Sample areas were always within 2 grid distances of the base of the wound epidermis (1 grid distance = 0.2 mm) prior to blastema formation and, after blastema accumulation, throughout the blastema area. This sampling technique is based on that of Kelly and Tassava[8]. At least 300 cells were counted in each limb. The mitotic indices (MI) were calculated in the following way:

$$\mathrm{MI} = \frac{\text{number of cells in mitosis}}{\text{total number of cells}} \times 100.$$

Any cell in late prophase through telophase was considered a mitotic figure. Left limbs were tested for sensitivity and movement throughout the post-denervation period. Blastemas were staged throughout the regeneration period[9]. To confirm denervations and to determine the rate of nerve ingrowth, some sections of each limb were stained for nerves[10].

Results and discussion. As shown in figure 1, the mitotic index increased in control, right limbs beginning on day 3 post-amputation and rose to over 3% in some blastemas. Blastemas passed through early bud, mid bud, late bud, and palette stages[9] from days 5 through 8, after which differentiation began and mitotic indices decreased. By day 12, 4 digit regenerates were present and mitotic indices dropped to below 1%. In contrast, the denervated, left limbs showed no significant increase in the mitotic index through the same period of time (figure 1) consistent with earlier findings. Dedifferentiated cells could be seen in the distal regions of the denervated limbs beginning on day 3 post-amputation (figures 2 and 3) but mitoses were rarely observed and blastemas did not develop.

Earlier investigators noted similar dedifferentiation in denervated limbs[2,4] and pointed out that in no case should the small distal accumulation of dedifferentiated cells (figures 2 and 3) be called a blastema since resorption is occurring[2]. The present results are consistent with earlier speculation on the mechanism of resorption[6]. During the denervation period, muscle, connective tissues, and the distal ends of the radius and ulna undergo histolysis. The cells dedifferentiate, block in the cell cycle, and are slowly lost from the limb stump. Loss of internal tissues accounts for limb resorption and partial closure of the amputation surface by skin (figures 2 and 3). This can be noted by comparing the shorter lengths of the radius and ulna in figures 2 and 3 with the longer radius and ulna in figure 5. The dermis extends over the cut ends of the radius and ulna in denervated limbs (figures 2 and 3) whereas the dermis can be seen to be slightly proximal to the distal ends of the radius and ulna in control regenerating limbs (figure 5). The blastema occupies the entire width of the limb in these young, small larvae (figure 5) while in older and larger *Ambystoma* larvae, the blastema is restricted to a small area of the amputation surface[9]. Mitoses began in denervated

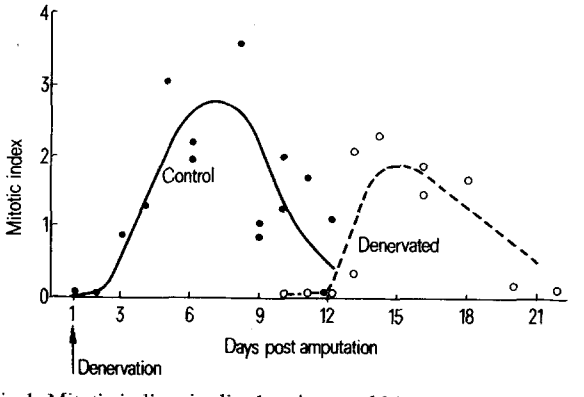

Fig. 1. Mitotic indices in distal regions and blastemas of innervated right limbs during normal regeneration and in denervated left limbs before and during re-innervation. Each point on the graph represents the mitotic index of 1 blastema or distal limb stump of a right, control limb (●—●) or a left, denervated limb (○—○). Denervated limb stumps did not show significant mitoses through the 3 to 10 day period of maximum mitoses in control limbs.

limbs upon re-innervation (figure 1) and essentially normal regeneration followed, as if the level of amputation was at the level of resorption[2]. Mitoses could be seen among the distal dedifferentiated cells beginning on day 13 (figures 1, 3 and 4). It is of interest that the histological appearance of the distal stump and the cytological appearance of the cells therein did not differ from days 10 through 13 (compare figures 2 and 3), however, mitoses could be seen among these cells only on days 13 and beyond (figure 5). It is possible that although dedifferentiated cells are blocked in the cell cycle and lost from the limb stump under denervation conditions[2,4], re-innervation can 'rescue' at least some of the population. Why the mitotic indices of denervated, re-innervated blastemas only reached 2.5% instead of the

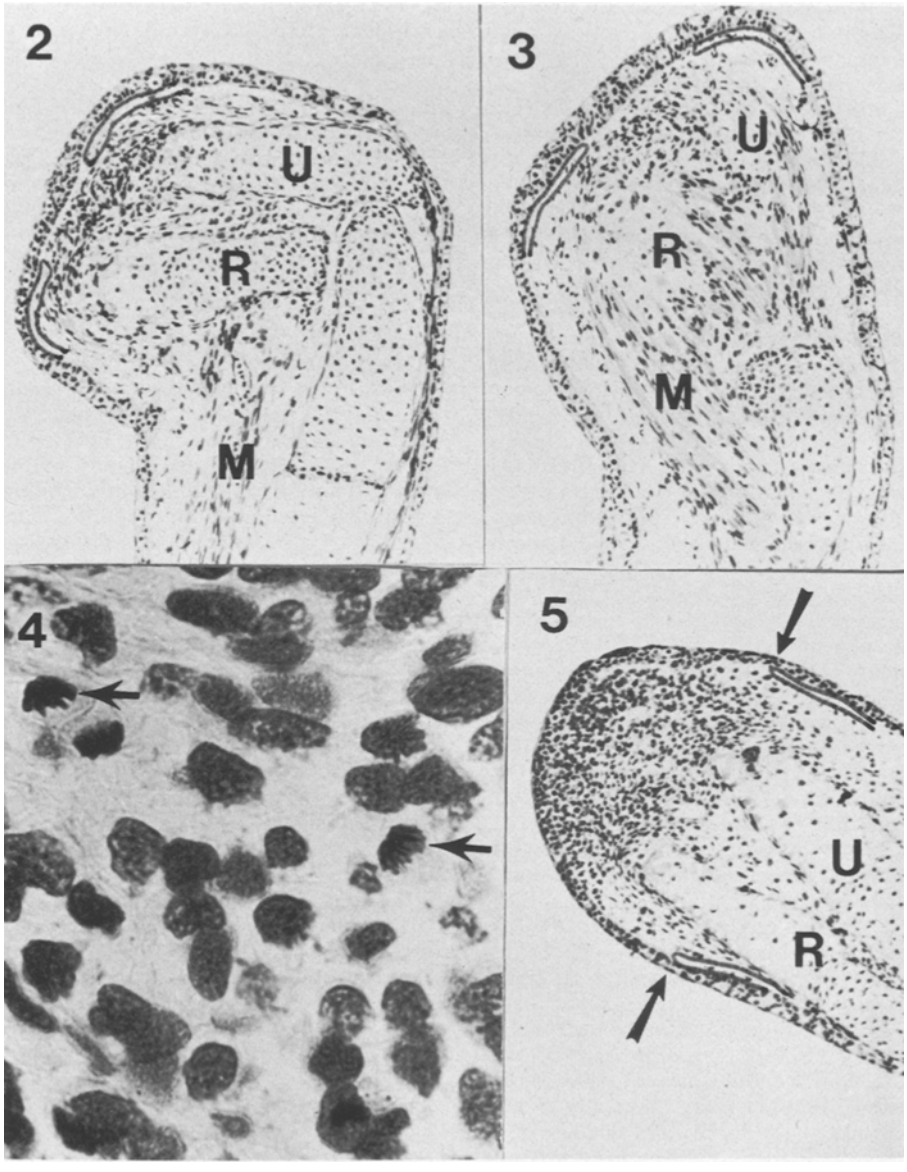

Fig. 2. A low power micrograph of a longitudinal section through a left forelimb denervated 1 day post-amputation and fixed 12 days post-amputation. No mitotic figures were observed in this limb. Skin has partially enclosed the amputation surface as the internal tissues have been lost by resorption. The dermis is outlined in ink to call attention to the fact that wound epidermis is still present at the distal tip. The cells distal to the radius and ulna and underneath the wound epidermis appear histologically to resemble the cells of the blastema of control regenerating limbs and are also similar to cells of denervated limbs on day 13, by which time abundant nerve fibres have reached the distal region of the limb (see figure 3). U, ulna, R, radius, M, muscle. Feulgen. ×40. Fig. 3. A low power micrograph of a longitudinal section through a left forelimb denervated 1 day post-amputation and fixed 13 days post-amputation. This section is slightly lateral to the section of figure 2 and shows some of the muscle (M) of the lower arm. This limb resembles the limb of figure 2 in all respects except that mitotic figures (MI = 2.4%) are present among the dedifferentiated cells at the tip. Many nerve fibres are present among the dedifferentiated cells of day 13 and 14 limbs (see figure 6). A higher magnification of these mitotic cells is seen in figure 4. Within 2 to 3 days, a medium bud stage blastema would be developed on this limb, resembling the control blastema of figure 5. U, ulna, R, radius. Feulgen. ×40. Fig. 4. A high power micrograph of a portion of the section in figure 3 which shows that on the basis of cytological features, the cells in denervated limbs resemble cells of innervated limbs, even at blastema stages. Distal regions of denervated limbs showed abundant nerve fibres on days 13 and 14 post-amputation (see figure 6) correlated with increases in the mitotic indices. Several mitotic figures (arrows) can be seen. Feulgen. ×960. Fig. 5. A low power micrograph of a longitudinal section through a right forelimb fixed 6 days post-amputation at the mid-bud stage of regeneration. The mitotic index of this blastema was 2.3%. Note the edges of the dermis (arrows) somewhat proximal to the cut ends of the radius and ulna. The distal ends of the radius and ulna have undergone some histolysis but no significant resorption has occurred. The blastema covers the entire width of the limb in these small *Ambystoma* larvae. Feulgen. ×40.

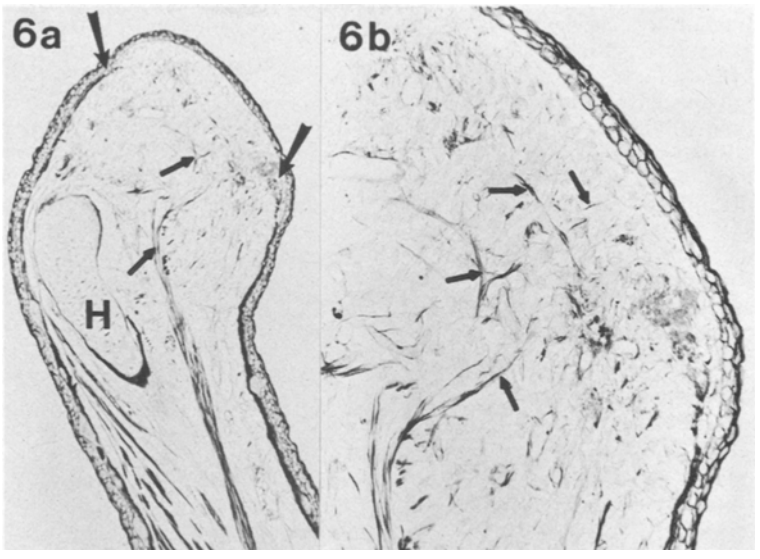

Fig. 6. Micrographs of a longitudinal section stained for nerves through a left forelimb denervated 1 day post-amputation and fixed 14 days post-amputation. The section in *a* is slightly lateral to the radius and ulna. Abundant nerve fibres [small arrows] in the area of dedifferentiation are correlated with an increase in mitosis at this time. The area of wound epidermis is somewhat restricted by the edges of the dermis [large arrows]. H, humerus. Samuel's nerve stain. *b* is a higher magnification of a portion of the section in *a*. a, ×40. b, ×100.

3.5% maximum of control blastemas is not known. The regeneration time for left limbs, days 13–22, was the same as for controls, days 3–12 (figure 1).

Denervated limbs showed movement on days 10 and 11 and sensitivity to touch on days 12, 13, and 14, suggesting that nerves were at the tip of the limb at the time mitosis began. Nerve staining confirmed this view. Those limbs on days 12, 13, and 14 which showed increases in the mitotic index also had abundant nerve fibres coursing among the dedifferentiated cells in the distal region of the limb (figure 6). Most important, ultrastructural examinations of denervated limbs at the time of re-innervation, during the 12 to 14 day period established in this study, will make it possible to test whether nerves make contact with dedifferentiated cells before cycling can begin.

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2 E.G. Butler and O.E. Schotté, J. exp. Zool. *88*, 307 (1941).
3 C.S. Thornton, J. exp. Zool. *122*, 119 (1953).
4 R.A. Tassava, L.L. Bennett and G.D. Zitnik, J. exp. Zool. *190*, 111 (1974); A.L. Mescher and R.A. Tassava, Devl. Biol. *44*, 187 (1975); R.A. Tassava and A.L. Mescher, J. exp. Zool. *195*, 253 (1976).
5 R.A. Tassava and A.L. Mescher, Differentiation *4*, 23 (1975).
6 R.A. Tassava and W.D. McCullough, Am. Zoologist *18*, 729 (1979).
7 M. Singer, Q. Rev. Biol. *27*, 162 (1952).
8 D.J. Kelly and R.A. Tassava, J. exp. Zool. *185*, 45 (1973).
9 P.W. Tank, B.M. Carlson and T.G. Connelly, J. Morph. *150*, 117 (1976).
10 E.P. Samuel, Anat. Rec. *116*, 511 (1953).

Inhibition of neutrophil-mediated cytotoxicity by α2 macroglobulin[1]

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Summary. Highly purified human α2 macroglobulin (1.2–10 mg/ml) was shown to inhibit phytohemagglutinin-induced or antibody-induced lysis of chicken erythrocytes by polymorphonuclear neutrophils. Inhibition was not associated with impaired contact between effector and target cells but rather with the antiprotease activity of α2 macroglobulin.

Many of the tissue lesions in Arthus reactions are mediated by lysosomal enzymes, particularly acidic proteases, released by polymorphonuclear (PMN) neutrophils upon ingesting antigen-antibody complexes. In the absence of phagocytosis, PMN which encounter immune complexes deposited upon a solid matrix extrude lysosomal enzymes by a mechanism of reverse endocytosis or frustrated phagocytosis[2]. 2 in vitro models have been designed to investigate the mechanisms of these reactions. Basically they use non-phagocytable ^{51}Chromium-labelled target cells, e.g. chicken erythrocytes, which are incubated with PMN. Precise measurement of target cell lysis is achieved by determination of ^{51}Cr-release into the supernatant. The cytotoxic reaction is triggered either by agglutinating lectins[3] or by anti-target cell IgG antibodies[4] which bind to PMN surface receptors[5]. Little is known of the various mechanisms which may control the in vivo counter-parts of such cytotoxic reactions. We have investigated the possible regulatory role of α2 macroglobulin (α2 M), one of the major plasma protease inhibitors, the serum levels of which are known to rise during inflammatory reactions.

Material and methods. Human PMN were obtained from normal donors. Leucocytes were separated by sedimentation of heparinized blood on Dextran (Pharmacia Fine Chemicals, 5.04% solution, 24 vol.) - Isopaque (Winthrop, 4 vol.) followed by centrifugation on Ficoll-Isopaque as already described. PMN were collected in the pellet; they were freed of erythrocytes by hypotonic lysis, then washed and resuspended in RPMI 1640 medium supplemented with antibiotics and 2% fetal calf serum. Chicken red blood cells (CRBC) obtained from white Leghorn, 2–6-month-old, were labelled with ^{51}Chromium ($NA_2\ ^{51}CrO_4$, C.E.A. Gif-sur-Yvette) as described elsewhere[6]. Highly purified fractions of α2 M were obtained from Drs Bonneau and Latour (Institut Mérieux, Marcy l'Etoile). They were prepared from pooled human plasma by Rivanol precipitation