VITAMIN - A AND LIMB REGENERATION IN STAGES OF THE EGYPTIAN TOAD, BUFO REGULARIS
REUSS-HISTOLOGICAL STUDY

By
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فيتامين أ، والتجدّد الطرفي في أطوار العلوج المصري، بروف ريجولارس
رووس - دراسة نسيجية

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اختص هذا البحث بدراسة تأثير فيتامين أ، على تجدّد الطرف الخلفي لطورين بروف الريتين للعلوج المصري (برمائيات أنيزية) وذلك بعمل فحوص نسيجية لكل من بورقات مجموعة ضيقة وبرونيات مجموعة معالجة بفيتامين أ (تركيز 30 ويمحة دولية/ملليجرام) لمدة ثلاثة أيام بعد التعرض عند منتصف الساق.

وقد لوحظ حدوث بعض التغييرات النسيجية في أطوار المجموعة المعالجة بالفيتامين مقارة بأطراف المجموعة الضيقة، وذلك على مرحلتين: (1) أثناء المعالجة بالفيتامين، (2) بعد توقف المعالجة. وفي المرحلة الأولى، لوحظ مايلي: ظهور تعرج واضح في بشرة الجلد التي غطي الجزء المبطن من الطرف، عدم تغيير الغشاء القاعدي أفلس البشرة، حدوث نزيف دموي داخلي عند مستوى البتر، وعدم تكون خلايا البلاستيمما في المرحلة الثانية. استمر ووجه التجاعيد في بشرة الاجزاء لمدة أيام بعد توقف المعالجة بالفيتامين. كما ظهرت بعض خلايا البشرة منتفخة نتيجة وجود فجوات واضحة بها، كذلك تكونت بلاستيمما كبيرة الحجم نتيجة حدوث تكاثر خلايا نشط خصوصاً في الأطراف المنتهية إلى الطور المبكر. وقد لوحظ أن خلايا البلاستيمما المتكونة كانت في أغلب الأحوال أكثر تراكماً في المنطقة الخلفية للطرف عنها في المنطقة الأمامية.

وتم أيضاً قياس حجم البلاستيمما لكل من المجموعة الضيقة والمجموعة المعالجة بالفيتامين في كل من الطورين الريتين وعمل مقارنة بينهما لتاكيد النتائج التي تم الحصول عليها بهذا الحصص، يدعم نتائج هذا البحث الدراسة المورفولوجية عن التجدد الطرفي والمعالجة بدات التركيز من الفيتامين (ميخائيل وآخرون، 1994)، والتي أوضحت تأخر البدء في التجدد وظهور تضاعف طريقي في بعض الحالات خصوصاً في الطور المبكر.

Key Words: Vitamin - A, Limb histogenesis, Regeneration, Anuran larvae, Retinol palmitate.
ABSTRACT

The histogenesis of regenerated limbs, of both control and treated \textit{larvae} with 30 I.U./ml of retinol palmitate for 3 days, was studied in two larval stages (viz. stages 53 & 54) of the anuran amphibian, \textit{Bufo regularis} Reuss. Some histological changes took place in the amputated limbs of treated larvae in two phases: (a) during treatment and (b) after cessation of treatment and thereafter. In (a): the wound epidermal superficial layer became corrugated, basement membrane was not discernible, local haemorrhage was extensive and blastema call accumulation was inhibited. In (b): the epidermal surface maintained its irregularity for some days and contained numerous large vacuolated cells. Also, cells from the dedifferentiated stump tissues underwent active proliferation leading to the establishment of a large blastema, particularly in the case of the earlier stage. Cellular condensations in these blastemas were mostly unevenly distributed; they appeared more closely packed together posteriorly than anteriorly along the antero-posterior axis. Histological observations concerning blastema cells and their active proliferation after cessation of treatment, were confirmed quantitatively through the blastema volume in both stages.

INTRODUCTION

Treatment with retinoids had adverse implications on the regenerative capacities of vertebrate limbs\cite{1-8}. Treatment might cause retardation in growth due to acute inhibition of cell division in the early blastema\cite{1-9}. On the other side, following treatment with specific dose for definite period, retinoids cause progressive dedifferentiation as well as proliferation of produced blastema cells; thereby improving the potential for blastema formation with greater morphogenetic capacities than usual\cite{10-13}. If differentiation does proceed, vitamin A can induce accelerated differentiation so that limb duplication may occur\cite{14-15}.

In the amputated axolotl limb, treatment with retinoids caused some histological peculiarities such as:

1-Consistent thickening of the epidermal covering along the posterior edge of the limb and high cell density of the formed blastema beneath this thickening so that the cells were more tightly packed along the posterior side than the anterior one\cite{16,18}.

2-Discernible irregularity in the surface morphology of the epidermal covering with conspicuous variation of the size of its cells and meanwhile induction of ciliated epithelia\cite{17,18}.

3-Direction of the growing regenerate towards the posterior side and not exactly along the longitudinal axis of the limb\cite{1,16,18}.

4-Distinct local haemorrhage adjacent to the wound area\cite{18}.

5-Permanent thickening of the basement membrane\cite{18}.

Since histological studies on anuran amphibians in that field are rather limited, the present investigation aimed primarily to follow up the basic sequential histological changes in the cells of the treated amputated limbs in larval stages of the anuran, \textit{Bufo regularis} and to compare the results with others so far known in the literature, on amphibians in general. The present work would also provide a deeper insight to explain the causal factors leading to production of limb duplication or side outgrowths, recently reported upon, after the use of the same concentration of retinol palmitate\cite{19}.

MATERIAL AND METHODS

Two larval stages of the Egyptian toad, \textit{Bufo regularis} Reuss, viz. stages number 53 and 54 (staging according to Sedra and Michael\cite{20}) were used for this study. Transection midway through the shank of the left hindlimb, of 30 tadpoles belonging to each of the two stages, was done as shown in Fig.1.

After amputation, half number of the larvae were immersed for three days in tap water to which retinol palmitate ("Arovit", Roche, India), dissolved in absolute ethanol, had been added at a concentration of 30 I.U./ml. The other half in each stage was kept for 3 days also in normal tap water to which the solvent had been added at the same concentration as in the treated cases (0.1ml absolute ethanol per liter). From the fourth day onwards, larvae were allowed to continue their development in normal tap water (for details see previous work\cite{19}). Rearing period from the time of amputation till the end of metamorphosis lasted 2\frac{1}{2}-3 weeks for the controls, and about 3\frac{1}{2}-4 weeks for the treated individuals.

Fixation of three specimens of both controls and treated animals was carried out in Bouin's fluid after 1,3,5,7 and 10 days post-amputation. Histogenesis was followed up in the limbs of these time-series, after longitudinally sectioning at 6 \mu m and staining with haematoxylin and eosin.

A comparative volumetric study was carried out on the blastemas of the time-series between controls and treated limbs after 3.5, and 7 days post-amputation. Such study was done by drawing the blastema using a camera lucida in every other section. By means of a graphic tablet (Apple Computer Inc.), the area of each drawing was determined and the total volume of the blastema of each specimen in each of the considered time-series was obtained by means of Computer programme (Apple IIe).

RESULTS

(A) Histogenesis of Stage 53-Larvae

(i) Controls

By the end of the first post-amputation day, the wound surface was covered by one layer of cuboidal epithelium
with a discernible apical knob. Elimination of free blood corpuscles, damaged cells and pigment granules took place through this apical knob. By the third post-amputation day, the wound epidermis was thickened greatly and reached several layers. Apical epidermal knobs were absent, and elimination of damaged cells and cellular debris seemed to be complete. The basement membrane was formed beneath the wound epidermis (Fig. 2). Dedifferentiation of distal stump cells as well as cellular proliferation were taking place giving rise to a well-developed blastema.

By the end of the fifth day, persevering proliferation of blastema cells had produced morphologically a considerable regenerative outgrowth restoring the missing shank half and base of the foot (Fig. 3). Histologically, however, relatively few newly differentiated procartilage cells, myoblasts and early dermal tissue cells, could be observed close to the amputation level with distally cells did not resume redifferentiation.

After seven post-amputation days, each of the three specimens studied showed clear indentation between all five toes of the regenerate (Fig. 4). Progressive redifferentiation of blastema cells was taking place in the outgrowth in a proximo-distal direction. By the end of the tenth post-amputation day, the regenerate appeared well-developed and the foot was supported by normal skeletal elements, including five metatarsals and primordia of phalanges (Fig. 5).

(ii) Treated cases

Some important histological changes were observed in the treated specimens in comparison with those of controls. By the end of the first day, i.e. during immersion of the operated larvae in the Vitamin A solution, the migrating wound epidermis took the appearance of an acute irregular or corrugated outline (Fig. 6) without formation of a recognizable apical knob.

After the post-amputation days, the epidermal covering possessed a distinct apical knob, capturing a large amount of cellular debris and blood corpuscles in numerous cysts (Fig. 7). No basement membrane was discernible in the specimens of this time-series. Cellular dissociation and dedifferentiation of distal stump tissue appeared to be just ensuing, without signs of a cellular population for blastema formation. By the end of the fifth day (2 days after cessation of retinol palmitate treatment), the epidermal cap appeared thicker than normal, probably due to existence of numerous large vacuolated cells which gave its surface a rather irregular appearance. The epidermal covering was underlied by a well-distinct basement membrane. The regenerate contained a large number of healthy mesenchyme-like cells forming an expanded blastema (Fig. 8), which showed many mitotic figures. Noteworthy, the proliferating blastema displayed asymmetry in its cellular distribution in which the cells appeared more aggregated in the posterior half than in the anterior one.

After seven post-amputation days, progressive dedifferentiation and active proliferation of the dissociated stump cells added more and more mesenchyme-like cells to the blastema. The more densely-packed blastema cells, along the posterior half of the extra-ordinary large blastema, appeared more pronounced than in the two earlier days. Large subcutaneous spaces were occasionally seen deeply in the regenerated outgrowth. In one specimen, belonging to this time-series, blastema cells were accumulated in two well-defined centers forming two partially separated blastemas (Fig. 9).

By the end of the tenth post-amputation day, rapid outgrowth accompanied by cellular differentiation had taken place in a proximo-distal direction. Large subcutaneous spaces were sometimes seen extending along the regenerate. In the shank region, the regenerate had restored the excised parts of the tibia and fibula, surrounded by several groups of muscle fibres, dermal tissue and pigment cells. The next limb part, however, showed condensation of procartilage, or still mesenchyme-like cells in one or two centers, that might form a normal ankle region or a duplicated limb segment.

(B) Histogenesis of Stage 54 Larvae

(i) Controls

As a general observation, the control limbs of this stage showed close similarity to those of Stage 53 during the first three days post-amputation. After five days, however, the well-formed blastema appeared usually in the form of a cone-like structure without showing any signs of redifferentiation.

By the end of the seventh post-amputation day, redifferentiation was resumed in a proximo-distal direction. The distal half of the shank as well as the foot region with usually three toe protuberances, were restored (Fig. 10).

After ten post-amputation days, the limb was perfectly developed in two specimens while the third specimen showed a four-toed regenerate. Histologically, chondrification of the newly formed pre-axial metatarsals as well as the digital elements completed the foot pattern.

(ii) Treated cases

By the end of the first day i.e., during immersion of the operated tadpoles in the retinol palmitate solution, the surface of the migrated epidermal cap was quite irregular and showed no apical knob. However, several small epidermal tongues from the wound epidermis were seen intruded into...
the cut stump area where clusters of blood corpuscles, damaged cells and pigments were detectable.

By the end of the third post-amputation day, the epidermal tongues appeared more recognizable while the basement membrane was still absent apically (Fig. 11). Below the epidermal cap, very few mesenchyme-like cells were detectable.

By the fifth post-amputation day, cellular debris, free blood corpuscles, and damaged cells appeared to be completely eliminated from the wound area. Some of them, however, were still found captured between the epidermal cells on their way to be exteriorized. A distinct basement membrane was formed underneath the epidermal cover, which exhibited an irregular surface and contained large vacuolated cells. Large number of mesenchyme-like cells, derived from the severed stump tissues, had formed a well-developed blastema with many mitotic figures. As the case of the analogous time-series of Stage 53, the blastema cells were more densely packed in the posterior half than in the anterior one (Fig. 12).

By the seventh post-amputation day, the regenerate had restored morphologically the distal half of the shank and base of foot. From the histological point of view, the cellular bulk of the regenerate was composed mainly of still undifferentiated blastema cells.

After ten post-amputation days, the degree of cellular differentiation and organization depended upon the quality and mass of the blastema. Usually, chondrification of the restored parts of the zeugopodial elements, the proximal tarsalia and basal foot elements took place.

(C) Blastema Volume

The average volume of blastema was determined in control-and treated limbs of both stages studied after 3, 5, and 7 post-amputation days (three specimens for each time-series, see Material and Methods). The data obtained are shown in Table 1 and Fig. 13. They clearly indicate that blastema volume underwent clear suppression during treatment, but increased more profoundly after cessation of treatment in the earlier stage (Stage 53) than in the other one (Stage 54).

Table 1

Average volume of blastemas given in $\mu m^3 \times 10^6$ in both control and treated cases of Stage 53 and Stage 54 during early post-operative days

<table>
<thead>
<tr>
<th>Blastema Volume</th>
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<tbody>
<tr>
<td>Stage Number at the Time Of Amputation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>Treated (30 IU/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3d</td>
<td>5d</td>
<td>7d</td>
</tr>
<tr>
<td>Stage 53</td>
<td>8</td>
<td>11</td>
<td>0*</td>
</tr>
<tr>
<td>Stage 54</td>
<td>3d</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>5d</td>
<td>12</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>7d</td>
<td>0*</td>
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* substantial redifferentiation of blastemal cells took place in the regenerate, giving rise to distinct mesodermal tissues in a proximo-distal direction.
DISCUSSION

The present study revealed some important histological changes in the regenerating limbs of the retinol palmitate-treated larval axolotls in comparison with those of controls. As regards the wound epidermis, resulting after rapid migration of stump epithelial cells [21], the treated limbs displayed during the early post-amputation days formation of a conspicuously large apical knob in Stage 53-larvae, and production of several epidermal tongues intruding deeply into the stump tissue in Stage 54-larvae. Noteworthy, existence of apical knobs or epidermal tongues persisted in the treated larvae about two days more than in controls, indicating probably the degree of severe damage in the cut area as a result of exposure to retinol palmitate.

It is probably convincing that epidermal tongues play a fundamental role in cleaning the wound area from cellular debris, and this cleaning may be brought about by direct exteriorization, by digestion or through the blood circulation itself [22,23]. Therefore, it could be concluded that the exogenously applied Vitamin A caused more cellular damage in a stage-dependent manner.

In the treated limbs of both stages studied, the surface of the wound epidermis displayed an irregular or corrugated outline and numerous large vacuolated cells were clearly observed during the early post-operative days. The epidermal covering was also thickened apically, and the base membrane underneath it seemed to be relatively thicker than normal (Figs. 8 & 12). Irregularity of the epidermal covering, great variation in its cell size, as well as thickening of the basement membrane induced by Vitamin A treatment are consistent with the results of Scadding [17, 18] on the axolotl. However, Scadding [17, 18] noticed also a remarkable epidermal thickening along the posterior side, and production of ciliated cells (as a sort of metaplasia caused by retinoids).

Concerning the stump mesodermal tissues, the treated limbs in both larval stages showed existence of numerous scattered and clustered blood cells in the wound area during the early post-amputation days; a sign of extensive local haemorrhage. This observation is concordant with that of Scadding [18] in regenerating axolotl limbs under the influence of hypervitaminosis A. The treated limbs also had dilated subcutaneous spaces, a phenomenon previously reported upon by many authors after excessive Vitamin A treatment and represents probably a state of hydroema [11,24,25,27].

The present investigation showed that initiation of dedifferentiation of stump tissue cells and their proliferation of the ultimate establishment of blastemas in the treated limbs, were greatly inhibited during Vitamin A treatment. However, after 4 days of the treatment cessation (by the seventh post-amputation day), fairly large blastema has appeared (see Fig.13). Thus, proliferation of the blastema cells was triggered after cessation of retinol palmitate treatment, and changed from the regressive state to the progressive one and was enhanced conspicuously to supercede the normal limb regenerative abilities of the concerned stage. This behavior and sequence of blastema formation was reported upon by many authors [1,2,4,5,6,9,18]. The remarkable retardation, during treatment, was found to be due to acute inhibition of cell division [1,9]. The latter author revealed that retinoic acid treatment reduced mitosis in the regenerated axolotl limb some 68%, and retardation of size was corresponding to the reduction in the mitotic activity. However, inhibition of cell division is virtually a well recognized effect of retinoids on a wide variety of cell types [27,28].

The present work revealed that the active mitotic division, in the progressive stage, led principally to a pronounced increase in the cellular density in the regenerate, especially in the limbs of the earlier stage (compare Fig.8 with Fig.12). So, it is likely that efficacy of Vitamin A on mitotic rate varies and seems to be stage-dependent. The above mentioned conclusion is in harmony with the results obtained in a previous work [19] which revealed that treatment of larvae of the considered two stages, with the same dose of retinol palmitate, caused enhancement of regeneration in many cases (30%) of Stage 53-larvae, but in few cases (2%) of Stage 54-larvae. This enhancement was expressed in production of limb duplication, either along the proximo-distal axis or in the form of side outgrowths.

It is recorded in the literature that retinoids and their derivatives cause proximalization of the blastematous cells as well as posteriorization and ventralization in work based mainly on urodeles [8,15]. In accordance with this suggestion, Scadding [17,18] in the treated axolotl limbs marked asymmetry in the cellular content of their regenerating blastemas, with higher cellular density in the posterior side than in the anterior one. Such asymmetry of the blastema has been observed also in the present study in the larvae of the Egyptian toad, where the cells appeared more densely packed in the posterior half than in the opposite anterior half. Concerning the antero-posterior axis, some differences have been discovered recently in amphibian limb blastemas between the anterior and posterior halves. For example, in the normal regenerating axolotl limbs the concentration of retinoic acid in the posterior region of the mid-cone blastema is about 5 times higher than the level in the anterior region [7]. In adult *Xenopus laevis*, however, no antero-posterior gradient of retinoids was observed in the normal (non-treated) regenerating limb blastemas, which possess a limited regenerative capacity [7].

Possibly, the more cellular density in the posterior half of the blastema, observed in the vitamin A-treated limbs of axolotls [17,18], may indicate a positive correlation with the posteriorly directed outgrowth of blastema in these limbs. In case of the treated limbs [1,16,18] of the Egyptian toad, however, no distinct deviation of the regenerate from the
normal direction has been observed along the proximodistal axis. It is possible that there is in this anuran species a relationship between the observed cellular aggregation in the posterior half of the treated blastema and the mode of limb ontogenesis, during which tissue differentiation, at least in the foot area, begins in the post-axial region and proceeds pre-axially [20,29,30]. Eventually, further studies are needed to elucidate the significance of this dissimilarity in the blastemal cellular density in the Vitamin A-treated limbs.

Finally, it was observed in the present work two partially separated blastemas in a case belonging to Stage 53 (see Fig. 9). It is reported that there is a direct correlation between the limb pattern that forms and the regulation of blastema cell proliferation [21]. Accordingly, and in agreement with Jangir and Niazi [11], production of two blastemas may be a preliminary step towards generation of a lateral supernumerary outgrowth.

LEGEND OF FIGURES

Fig. 1 Schematic drawing of the operation illustrating the hindlimbs of stages 53- and 54-larvae. After amputation through the middle of the shank (line a), half number of the larvae were kept 3 days in retinol palmitate solution (treated cases), the other half in water medium in which the solvent has been added (controls). The operated tadpoles were reared in normal tap water till the time of fixation.

Figs. 2-5 Longitudinal sections through control limbs amputated at stage 53.

Fig. 2 Three days post-amputation. The wound epidermis (WE) is greatly thicker than normal. A relatively well-developed blastema (BL) is evident and a discontinuous basement membrane appears beneath the epithelial covering. Scattered melanophores (ML) are found under the wound epithelium. The crural nerve (N) runs along the anterior side of the stump. X 250.

Fig. 3 Five days post-amputation. The regenerate had restored the removed shank half and the foot base (FB) beyond the amputation level (double directed arrow). Abortive blastemal cell redifferentiation has formed chondroblasts, myoblasts (MB) and fibroblasts just distal to the amputation plane. Subcutaneous spaces (SS) are extended along the regenerate (f: fibula). X 150.

Fig. 4 Seven days post-amputation. Primordia of all five toes, covered with normal skin, are pronounced. The two tarsal elements, astragalus (A) and calcaneum (C), as well as post-axial metatarsals (MT) and digital elements (DE) are differentiating in a proximo-distal direction. Notice also differentiation of new muscle fibres adjacent to the tarsal elements. X 250.

Fig. 5 Ten days post-amputation. A perfect limb is now regenerated (the base of the fifth toe could be seen). Digital phalanges (PH) are differentiating in a postero-anterior (t: tibia, f: fibula). X 75.

Figs. 6-9 Longitudinal sections through treated limbs amputated at stage 53.

Fig. 6 One day post-amputation. A large number of cellular debris (CD) and blood corpuscles (BC) are present under the wound epidermis (WE) which displays an irregular surface. The basement membrane (BM) is evident underneath the stump epidermis only. X 75.

Fig. 7 Three days post-amputation: The migrated wound epidermis has formed a large apical knob (K). The knob contains numerous cysts filled with blood corpuscles and cellular debris (CD). Notice dilation of blood capillaries (BC). X 600.

Fig. 8 Five days post-amputation. The thickened epidermal covering is corrugated in outline and contains numerous large vacuolated cells. A distinct basement membrane is discernible underneath the epidermal cap. A large blastema (BL) is formed. Notice the asymmetry of the blastema where its cells are more densely packed in the posterior side (P) than in the anterior side (A). X 600.

Fig. 9 Seven days post-amputation. The regenerate here shows two, partially separated blastemas (BL & BI). One of them (BL) is projecting laterally and will form likely a supernumerary outgrowth. Notice presence of melanophores (ML) and dilated subcutaneous space (SS). X 400.

Fig. 10 Longitudinal section through a control limb amputated at stage 54, seven days post-amputation. Primordia of the post-axial toes are regenerated. Differentiation of the blastema takes place in a proxima-distal direction (ML: melanophore; MT & MT metatarsal of the third and the fifth digits). X 320.

Figs. 11 & 12: Longitudinal section through treated limbs amputated at stage 54.

Fig. 11 Three days post-amputation. Several epidermal tongues (TG) are intruding deeply into the cut stump area, containing damaged cells and blood corpuscles. Many free blood cells (BC) and cellular debris are still present in the distal region of the stump. Few disassociated and undifferentiated cells appear under the wound epidermis. The level of amputation is indicated by the double directed arrow. X 280.

Fig. 12 Five days post-amputation. The epidermal surface is irregular in outline. Some large vacuolated cells are present in the epidermal cap and the basement membrane (BM) is clearly distinct underneath. A large blastema (BL)
is formed. Notice asymmetry of the blastema with more cellular density in the posterior side (P) than in the anterior side (A). X 450

Fig. 13 This figure illustrates the quantitative analysis of blastema volume shown in table 1. Notice the obvious retardation of blastema establishment in the retinol palmitate

REFERENCES


