Histological effects of vitamin A on the tail amputated tadpoles of *Bufo melanostictus*

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Vitamin A has manifold effects on the development, growth and pattern formation of several amphibians. At the same time it causes severe embryonic malformations. The histological changes brought about by vitamin A in the tail tissues of anurans are quite amazing. A common morphological change brought about by vitamin A in tail-amputated tadpoles of *Bufo melanostictus* include the formation of a large bulbular mass at the distal end of the tail following tail regeneration. Histology revealed that the bulbular mass consisted of notochordal cells only. Other histological changes are: a thickening of the epidermis and the basement membrane, enlargement of the notochord and the nerve cord, thickening of the sheath covering the notochord and the myelin sheath covering the nerve cord and the disorganization of muscle bundles. The significance of such changes is discussed.

1 Introduction

Vitamin A, first discovered in 1909 (Pawson 1981) and compounds of related structure and function (the "retinoids") are teratogens. When administered exogenously to animals, they cause a variety of defects of morphogenesis and embryonic development (Kochhar 1967; Rosa *et al* 1986). Niazi and Saxena (1968) first observed that vitamin A has both inhibitory and modifying influences on tail regeneration in the tadpoles of *Bufo andersonii*. Since then several workers have started probing the effects of vitamin A on development and regeneration (Niazi and Saxena 1979; Sharma and Niazi 1979; Maden 1982, 1983; Scadding 1983; Thoms and Stocum 1984; Mohanty-Hejmadi *et al* 1992; Mahapatra 1993; Mahapatra and Mohanty-Hejmadi 1994) and have reported several interesting results. Perhaps the most dramatic effect seen hitherto has been the homeotic transformation of tails to limbs in *Uperodon systoma* (Mohanty-Hejmadi *et al* 1992). Although extensive work has been done on the histological effects of vitamin A on limb regeneration and limb development (Scadding 1983; Johnson and Scadding 1991), the histological effects of vitamin A on tail regeneration have not been dealt with. Little information is available on the histological effects of vitamin A; it is restricted to the tails of *B. andersonii* (Niazi and Saxena 1968) and *Rana temporaria* (Maden 1993). The detailed histological changes occurring during tail regeneration following vitamin A treatment have not been studied although Mahapatra (1993) carried out a morphological study on the effect of vitamin A on tail regeneration of the tadpoles of *B. melanostictus* and has reported observing the regeneration of ectopic limbs from the cut end of the tail; also, vitamin A partially suppressed the process of regeneration but did not inhibit it completely. To understand the nature of the observed vitamin A mediated morphological changes it is necessary to examine the histological changes underlying the morphological variations. The present work reports on study of the histological changes in amputated tails of *B. melanostictus* (Anura: Bufonidae) under the influence of vitamin A.

2. Materials and methods

Egg masses of the common Indian toad *B. melanostictus* were collected from the Utkal University campus during

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the rainy season and raised in the laboratory up to the hind limb bud stage measuring 8–10 mm (Mohanty-Hejmadi *et al* 1992) following the standardized procedure of Mohanty-Hejmadi (1977). Prior to and during the course of the experiment the tadpoles were fed with boiled egg and *Amaranthus* leaves *ad libitum*. For the study of histology, the tadpoles were anaesthetized in a 1 : 400 solution of MS 222 (tricaine methanesulfonate), amputated in the middle of their tails and reared in conditioned water containing 10 IU/ml vitamin A (Arovit, Roche) for 72 h (3 days). The controls were also reared in a similar manner but without vitamin A treatment. Following treatment, the tadpoles were removed, the regenerated tails were again amputated and fixed in aqueous Bouin's fluid on days 1, 2, 3, 4, 5, 6, 11, 16, 20, 27 and 30 (i.e., both during and beyond the period of treatment). The controls were also fixed on the corresponding days. The fixed tissues were then embedded in molten paraffin (m.p. 58°C–60°C), serially sectioned either longitudinally or transversely as necessary at 10 μm thickness and stained with Mallory's triple stain for examination in the light microscope. Four tadpoles from the experimental set and two from the control set were sectioned each time for histological study. All the four tadpoles fixed from the experimental group possessed similar morphological characteristics.

3. Results

Day 1

In both control (figure 1) and the treated tails (figure 2) a mass of dead cells and blood vessels were found at the extreme distal end 24 h after the initial amputation. A thin apical epidermal cap had formed at the distal end. The epidermis was single-layered. The notochord was constricted in both control and treated tadpoles (figure 2). The nerve cord was vesicular. Muscles were absent.

Thus there was no difference between the control and the treated tails by day 1 post-amputation.

3.2 *Day 2*

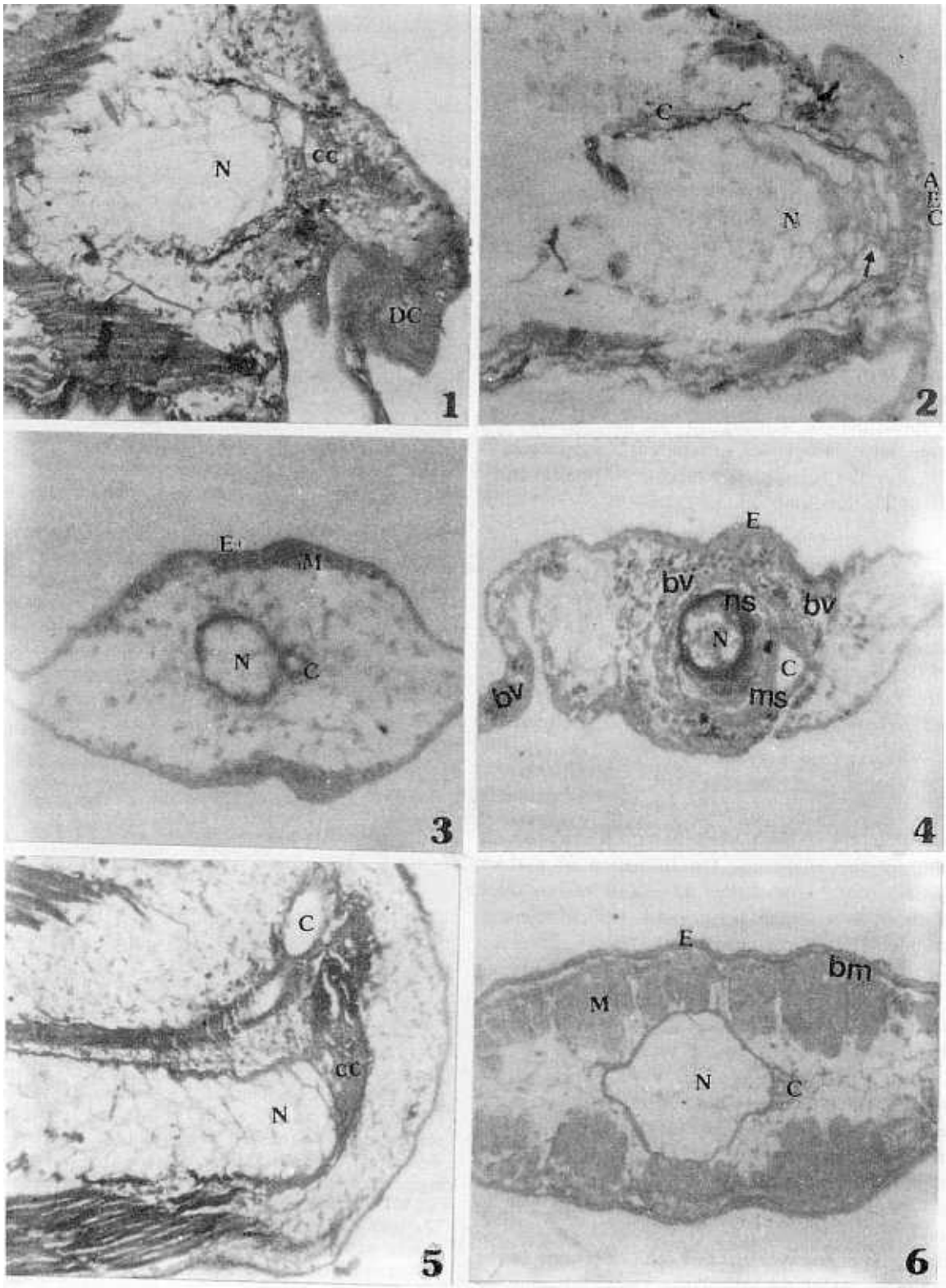
In the control, the epidermis was single-layered. The AEC was no longer visible. The constriction of the notochord had disappeared. The nerve cord was small and had become normal. Muscles were beginning to redifferentiate and were arranged in small bundles on the lateral sides. However, in the treated tails, no significant change was observed and all the characters remained the same as in day 1.

3.3 *Days 3–6*

In the control, the epidermis was single-layered. By day 3, the notochord, nerve cord and muscles had all become normal (figure 3). In the treated tails, the epidermis was multilayered. The basement membrane was present but discontinuous. The chorda cells were compact. The notochord was covered by a thick notochordal sheath (figure 4). Blood vessels were found in plenty. These were probably the subepidermal vascular pools. By day 6, the notochord was still constricted. The chorda cells were in the process of redifferentiation and reorganization as many undifferentiated chorda cells could be still seen at the distal end of the notochord. The nerve cord was enlarged, vesicular and covered by a thick myelin sheath. Muscles were still dedifferentiated (figure 5).

After amputation the chorda cells become dedifferentiated up to day 1. In the control tadpoles, the cells become redifferentiated by day 2 when regeneration is almost complete and by day 3 all the cells have redifferentiated completely. But in the treated tadpoles, by day 6 the chorda cells of the notochord were in the process of redifferentiation.

Figures 1–6. Longitudinal section of tail of *B. melanostictus* (control) 1 d post-amputation. The notochord (N) is dedifferentiated: some chorda cells (cc) are seen in a dedifferentiated state. A large number of dead cells (DC) are seen in the extreme distal end. These have probably oozed out to close the wound surface just after amputation (× 150). (**2**) Longitudinal section of tail of *B. melanostictus* (treated) 1 d post-amputation. A very thin apical epidermal cap (AEC) is present at the distal end of the tail. The notochord (N) is constricted (marked by arrow). The nerve cord (C) is vesicular. A similar AEC, a constricted notochord and a vesicular nerve cord were also found in the controls but are not visible in the particular section in figure 1 (× 150). (**3**) Transverse section of tail of *B. melanostictus* (control) 3 d post-amputation. The epidermis (E) is single-layered. The notochord (N), nerve cord (C) and muscles (M) have all become normal. The muscles have redifferentiated by day 3 and are arranged in very small bundles on the lateral sides just beneath the epidermis (× 150). (**4**) Transverse section of tail of *B. melanostictus* (treated) 3 d post-amputation. The epidermis (E) is multilayered. The notochord (N) is compact and covered by a thick notochordal sheath (ns). The nerve cord (C) is enlarged and covered by a thick myelin sheath (ms). Numerous blood vessels (bv) are seen in the section (× 150). (**5**) Longitudinal section of tail of *B. melanostictus* (treated) 6 d post-amputation. The chorda cells (cc) of the notochord (N) are still in a dedifferentiated state. The nerve cord (C) is enlarged and vesicular (× 150). (**6**) Transverse section of tail of *B. melanostictus* (control) 11 d post-amputation. The epidermis (E) is multilayered as the tadpole is approaching metamorphic climax. The basement membrane (bm) present just beneath the epidermis is prominent, conspicous and continuous. Muscles (M) are arranged in well defined bundles. The notochord (N) and nerve cord (C) are normal as usual (× 150).



Figures 1–6.

species of anurans *i.e. Polypedates maculatus* and *R. tigerina*, a thick and conspicuous apical epidermal cap (AEC) was found by day 1 post-amputation. The AEC persisted for almost 2 days (unpublished data). Though an AEC was found in *B. melanostictus* as well, it was not so prominent and persisted only for a single day—it was no longer seen by day 2. The small size of *Bufo* tadpoles in comparison to other anuran tadpoles might be a possible reason why the AEC was very thin (because a limited amount of tissue might have been available for its formation). The formation of an AEC is a common feature found in all amphibians. A similar AEC has also been observed by Scadding (1989) in the vitamin A treated amputated limbs of *Ambystoma mexicanum.*

The epidermis became multilayered in the treated tadpoles by day 3 and continued to remain multilayered until metamorphosis. The epidermal cells were uneven in shape and size. In the controls, a multilayered epidermis was seen from day 11 onwards. This is the time when the *Bufo* tadpoles are heading towards their metamorphic climax (see Mohanty-Hejmadi and Parida 1984). Yoshizato *et al* (1993) studied the transformation of epidermal cells of the tail of the anuran larva into the adult type. They found that in the earlier stages (up to stage XXI; Taylor and Kollros 1946) of larval *R. catesbeina* the tail epidermis contains two types of epidermal cells i.e., apical and skein cells. The basal cell type characteristic of the body epidermis is not found in the tail epidermis initially. At stage XXIII the basal cells also appeared between the skein cells in the tail epidermis. In the present study the multilayered epidermis observed in the treated tadpoles at an early stage was perhaps due to the multiplication of the skein cells which normally occurs at later stages. The increase in the thickness of the tail epidermis of the treated tadpoles was probably due to the interference of vitamin A. Scadding (1989) reported that when amputated limbs of *A. mexicanum* were treated with retinol palmitate, the epidermis showed a number of changes including the development of a very uneven and creviced surface, a great deal of variation in cell size and the development of ciliated cells on the surface layer of the epidermis.

The notochord became constricted at the site of initial amputation. The constriction persisted for one day in the controls and became normal by day 2 whereas in the treated, it remained for four days. The chorda cells were in the process of redifferentiation by day 4 because by day 3, some of the chorda cells were still in a dedifferentiated state. The notochord became enlarged and was enveloped by a thick notochordal sheath. The chorda cells in the extreme distal sections were compact so much so that individual cells were not discernible. A large bulbular mass was visible at the tip of the tail by day 27. The mass internally consisted of a massive amount of thin-walled, notochordal cells only.

During limb regeneration of tadpoles of *A. mexicanum* exposed to vitamin A, Maden (1983) observed that when the dedifferentiated cells were liberated at the tip of the amputated limb, the effect of cell division was manifest. As a result of the inhibition of cell division after vitamin A treatment the cells were clumped into a tight ball. This effect was presumably mediated via changes in the surface properties of the dedifferentiated cells. A preliminary investigation into the cellular effects of vitamin A indicated that though mitosis was severely reduced, it was not completely abolished. This revealed the paradoxical nature of vitamin A; it suppressed cell division, yet once released from this inhibition, extra pattern elements were regenerated (Maden 1983). During tail regeneration as in the present study, the cells present at the cut end of the tail absorb vitamin A and it is possible that vitamin A exhibits its paradoxical influence on the cells of the amputated tail tip. This might be a possible reason for the formation of compact chorda cells in the regenerating notochord on one hand and the formation of extensive chorda cells within the bulbular mass on the other hand.

Thus, vitamin A has two simultaneous effects; one of these effects might be to alter the cell cycle thereby suppressing the differentiation of chorda cells, and the other effect might be to alter pattern formation, thus inducing the formation of a large bulbular mass containing well-developed chorda cells only. The chorda cells were also found within the bulbular masses of *R. temporaria* (Maden 1993), *P. maculatus* and *R. tigerina* (unpublished data).

By day 1 postamputation, the nerve cord became vesicular in both the control and treated tails. This vesicular nerve cord persisted in treated tadpoles up to the emergence of forelimbs while it became normal by day 2 postamputation in the controls. An enlarged, vesicular nerve cord, covered by a heavy deposit of myelin in the form of a sheath suggested that vitamin A definitely interfered with the regeneration of the nerve cord thereby delaying the process and at the same time caused the deposition of extra myelin around it. The changes brought about by vitamin A leading to the thickening of the myelin sheath and formation of a vescicle at the distal end of the nerve cord in the present study is not known. Iten and Bryant (1976) have studied changes in different tissues in histological section of the adult newt *Notophthalmus viridescens* without vitamin A treatment. Within 2 days of amputation the ependymal epithelium of the spinal cord closes off to form a very small ependymal vescicle. In the present study also, once the vesicle of the nerve cord is formed in the treated tails soon after amputation, vitamin A retains the formation of the vesicle as a result of which it is unable to close off, unlikely as in the controls. This results in a vesicular nerve cord all throughout the course of the experiment.

Muscles were dedifferentiated too following amputation by day 1 in both the control and the treated tadpoles. By day 2 they had redifferentiated in the controls but in the treated tadpoles they remained in an unorganized state and were not arranged in well-defined bundles. Iten and Bryant (1976) have shown that amputation in the tail of the adult newt results in the transection of 1 or 2 of the V-shaped myomeres and by 2 or 3 days of amputation there are signs of muscle breakdown due to the disorganization of the striations in severed muscle fibres, some sarcoplasmic degeneration and muscle fragments with and without nuclei. In the present study also this explanation seems to be valid and offers a possible reason for the disorganization of the muscle bundles in the control tadpoles up to 2 days postamputation. In the treated tadpoles, following amputation, as the muscle fibres become dissociated vitamin A probably interferes among the striations thereby preventing bundle formation. As a result, the muscles become clumped and remain dissociated and unorganized (Das 1994). Clumping of muscles has also been observed in *P. maculatus* and *R. tigerina* (unpublished).

Niazi and Saxena (1979) have shown that among the three axial tissues (muscle, notochord and nerve cord) regeneration of muscles was the most adversely affected by vitamin A and notochord the least. The present study also showed that the notochord regenerated the earliest and the muscles the latest. The notochord does not dedifferentiate sufficiently to lose morphological identity at any time during tail regeneration. The spinal cord also do not dedifferentiate to lose morphological identity. Both of them redifferentiate earlier than the muscles. This indicates the differential susceptibilities of cells responsible for regeneration of three tissues to vitamin A. The differential effects of vitamin A on the development of notochord, nerve cord and muscles in the regenerating tail could be possibly related to the different degree of differentiation of cells originating from these tissues in the stump and to temporal differentiation in the beginning of their rehistogenesis (Niazi and Saxena 1979). The present study therefore confirms the observation of Niazi and Saxena (1979) that the notochord and the nerve cord regenerate earlier than the muscles.

Though the present result has offered possible explanations for some of the effects of vitamin A, it is still unclear how vitamin A treated animals retain a thick notochordal sheath and a thick myelin sheath around the notochord and nerve cord respectively. The basis of homeotic transformations—by what mechanisms tail tissues are converted to limb tissues in several species of anurans—remains unknown (Mohanty-Hejmadi *et al* 1992; Maden 1993; Mahapatra and Mohanty-Hejmadi 1994; Das and Dutta 1996). More detailed and extensive studies are clearly needed if we are to understand the puzzling effects of vitamin A on limb and tail regeneration.

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