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RESEARCH ARTICLE

ROLE OF ACID AND ALKALINE PHOSPHATASES DURING VITAMIN A INDUCED ABNORMAL TAIL REGENERATION IN THE TADPOLES OF THE INDIAN TREE FROG

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ABSTRACT

Treatment of vitamin A (10IU/ml for 72 hours) to the tail amputated tadpoles of the Indian tree frog, *Polypedates maculatus* led to regeneration of abnormal tails from 20% abnormally regenerated tails ectopic limbs developed. A biochemical investigation revealed an elevation in the specific activity of acid and alkaline phosphatase in the regenerating tail of the control group in comparison to the original tails. In the vitamin A induced abnormally regenerated tails, there was significant increase in the activity of these two enzymes than the respective regenerated tails of the control group. Thus, it is evident that vitamin A induced specific activity of both acid an alkaline phosphatase in the abnormally regenerated tails, a prerequisite for ectopic limb development.

INTRODUCTION

Regeneration is an interesting biological phenomenon where lost part of an organism is restored. Among tetrapods, amphibians exhibit the highest degree of regenerative ability. Urodels regenerate the lost part of their tails and limbs throughout life, whereas complete restoration of tail and limb is restricted to the larval period in anurans. However, exposure to exogenous vitamin A and its derivatives, the retinoids (retionol, retinal and retinoic acid) interfere with normal process of regeneration of limbs and tails in anurans tadpoles. The inhibitory and modifying influence of vitamin A on tail regeneration was observed for the first time by Niazi and Saxena (1968) in anuran tadpoles of *Bufo andersonii*. Subsequently, Scadding (1987) reported dose dependent inhibition of tail regeneration in *Ambystoma mexicanum* and *Xenopus laevis* following vitamin A (palmitate) treatment.

However, the most remarkable effect of vitamin A (palmitate) on anuran tail regeneration was the homeotic transformation of tails to limbs (Mohanty-Hejmadi *et al*., 1992) in the marbled balloon frog *Uperodon systoma* on exposing the tail amputated limb bud stage tadpoles to vitamin A 10IU/ml treatment for 24 to 144 hours. Following this initial finding, there were several reports on vitamin A induced inhibition of tail regeneration, ectopic limbs and pelvic region formation in other anurans (Mahapatra and Mohanty- Hejmadi, 1994; Das and Dutta, 1996; Maden, 1993; Muller *et al*., 1996). Histological investigation, has revealed marked histological similarities between normal and vitamin A induced ectopic limb buds of *Polypedates maculatus* (Mahapatra *et al*., 2004). Besides, a hyper oxidative stress condition has been reported in the vitamin A induced abnormally regenerated tails of *P. maculatus* (Mahapatra *et al*., 2002), a prerequisite for development of ectopic limbs (Maden, 1993; Mahapatra and Mohanty-Hejmadi, 1994). Investigation at molecular level describes up-regulation of retinoid receptors (RARα, RXRα and RXRβ) in the retinoid treated tail blastema of *Rana temporaria* (Maden and Corcoran, 1996). To explain the possible causes of homeotic transformation of tail tissues to pelvic girdle and limbs, it has been hypothesized that the exogenous retinoids (Vitamin A or retinoic acid) change the positional value of tail blastema to body flank and limb specific homeotic genes are expressed (Bryant and Gardiner, 1992; Maden, 1996). However, the precise mechanism that leads to trans-differentiation of tail to limbs is yet to be understood.

An elevation in the level of phosphatases (acid and alkaline) during normal tail regeneration in anuran tadpoles (Janqueira, 1950) and limb regeneration in Urodeles (Inoue and Suzuki, 1969) have been reported. Elevation in the activity of acid phosphatase has also been described in urodels during retinoic acid induced regeneration of duplicate limbs (Ju and Kim 1993; 1994). Moreover, regeneration in amphibians is known to be associated with histolysis of the underlying tissues (Carlson, 2005) and cell death during early stage of regeneration has been reported to be essential during normal tail regeneration in anuran tadpoles (Tseng *et al*., 2007). Acid phosphatase, a lysosomal marker enzyme is known to be associated with lytic activities during tail regression in anuran tadpoles (Mahapatra *et al*., 2011) and alkaline phosphatase, a

membrane bound enzyme is associated with undifferentiated pluriopotent stem cells (O'Connor, 2008). Since undifferentiated blastemal cells below wound epidermis is evident during tail regeneration (Mochii *et al*., 2007) and major portion of vitamin A induced abnormal tails, a pre requisite for transdifferentiation of tail to limb are packed with undifferentiated cells (Mahapatra *et al*., 2004), understanding the involvement of alkaline phosphatase during vitamin A induced abnormal tail regeneration in anurans is important. Moreover, investigating the role of acid phosphatase becomes equally important as lytic activities are associated with regeneration in anurans (Carlson, 2005) and urodels (Ju and Kim, 2010). Present paper describes the specific activities of acid and alkaline phosphatase during normal and vitamin A induced abnormal tail regeneration in the tadpoles of the Indian tree frog, *Polypedates maculatus* (Anura: Rhacophoridae).

MATERIALS AND METHODS

Chemicals

The chemicals used in this study were of analytical grade. Folin-Ciocalteaus reagent was obtained from Qualigens Fine Chemicals, Glindia Ltd, India; p-Nitrophenyl phosphate (pNPP) and para-Nitrophenol (pNP) was obtained from Sisco Research Laboratory, Mumbai, India. Bovine serum albumin (BSA) was obtained from Sigma chemicals Co.; USA. Vitamin A palmitate (Piramal Healthcare Ltd., India) tablets were used for the treatment and the stains used for histology were obtained from Loba (India). All other chemicals were of the highest purified grade available.

Tadpoles

Foam nests containing eggs of the Indian tree frog, *Polypedates maculatus* (Anura: Rhacophoridae) were collected from Utkal University campus, Bhubaneswar, located at 85° 53'E longitude and 20° 21'N latitude during the monsoon period (July-September). The hatchlings were reared in the laboratory following standard procedure (Mohanty-Hejmadi, 1977). The tadpoles were fed with boiled *Amaranthus* green and yolk of boiled egg *ad libitum* throughout the experiment.

Tail amputation and treatment

Hind limb bud stage tadpoles i.e. Gosner stage 26 (Gosner, 1960) were selected for tail amputation. Tadpoles were anesthetized with 1:400 solution of MS222 prior to amputation through the middle of their tails. Following amputation, the experimental tadpoles were treated with vitamin A palmitate 10IU/ml for 72 hours, the optimal treatment condition for ectopic limb development (Mahapatra *et al*., 2002) and then transferred to conditioned tap water. The control tadpoles were reared in conditioned water following tail amputation. The experimental tadpoles were reared for 15days including treatment period. Similar treated and control group tadpoles were reared for morphological investigation and kept under observation till onset of metamorphosis i.e emergence of fore limbs.

Biochemical Estimation

A pool of 50 tadpoles was taken for a single assay. The non amputated tails of limb bud stage tadpoles were considered as the original group. The regenerated tails of 5, 10 and 15 days post amputated tadpoles of the control and the vitamin A 10IU/ml treatment were taken for biochemical analyses as control and treated groups, respectively. A 10% (w / v) homogenate was made with 0.25M sucrose from the original and regenerated tails of the control and treated groups. The homogenate was kept in ice cold condition and used for the assays for phosphatases (acid /alkaline) and protein. Acid phosphatase activity was determined according to the method of Guha *et al*., (1974) with p- nitrophenyl phosphate as substrate. Alkaline phosphatase activity was determined according to the method of Garen and Levinthol (1960) with p-nitrophenyl phosphate as substrate. The protein content of the sample was assayed by the method of Lowry *et al*., (1951). The enzyme activity was expressed as µmol p-nitrophenol (pNP) formed / mg protein / min at 37°C. Each experiment was replicated five times.

Statistics

Statistical analysis was done after Steel and Torrie (1980). The ANOVA test to find out significant difference between means was calculated by Duncan's multiple range test Using SPSS package. The same superscripts over the bars in figures 2 and 3 represent data, which are not significantly different ($P>0.5$).

RESULTS

Morphological effects of vitamin A

Normal looking tails regenerated in the control tadpoles within 15 days of tail amputation while abnormal tails regenerated in the treated tadpoles. In 20% abnormal tails, bud like structures appeared which subsequently developed into ectopic hind limbs (Fig. 1).

Biochemical effects of vitamin A

Acid phosphatase

The specific activity of acid phosphatase (ACP) was found to be 2.68 ± 0.1 µmol p-nitrophenol (pNP) formed / mg protein / min in the original tails of the limb bud stage i.e. Gosner stage 26 (Gosner, 1960) tadpoles (Table 1). In the control group, the level of ACP was observed to be 3.33 ± 0.21 µmol pNP formed / mg protein / min in the 5days regenerated tails. The level of ACP was 3.17 ± 0.07 and 2.87 ± 0.04 µmol pNP formed / mg protein / min, respectively in the 10 and 15 days regenerated tails of this group. In the vitamin A treated tadpoles, the level of ACP remained higher than their respective controls (Fig.2). After 5, 10 and 15 days of tail amputation the level of ACP was estimated to be 3.69 ± 0.45, 3.72 ± 0.23 and 4.13 ± 0.12 µmol pNP formed / mg protein / min, respectively.

Alkaline phosphatase

In the original tails the specific activity of alkaline phosphatase (ALP) was found to be 1.11 ± 0.08 µmol p-nitrophenol (pNP) formed / mg protein / min (Table 1). In the regenerated tails of the control group tadpoles the level of ALP remained higher than the original tail. The level of ALP was estimated to be 4.18 ± 0.16, 5.47± 0.15 and 3.18 ± 0.05 µmol pNP formed / mg protein / min in the 5, 10 and 15 days regenerated tails, respectively in this group. In the treated group, level of ALP remained higher than the regenerated tails of the control group. In the 5 days post amputated regenerated tails, the level of ALP

Table 1. Specific activity of acid phosphatase and alkaline phosphatase in µmol p-nitrophenol (pNP) formed / mg protein / min at 37°C of the original and regenerated tails of the control and vitamin A (10IU/ml for 72 hours) treated tadpoles of *Polypedates maculatus*

(N=20)	Original	Control			Treated		
		5day	10day	15day	5day	10day	15day
Acid	2.68	3.33	3.17	2.87	3.69	3.72	4.13
phosphatase	± 0.1	± 0.21	± 0.07	± 0.04	± 0.45	± 0.23	±0.12
Alkaline phosphatase	1.11	4.18	5.47	3.18	7.43	10.56	9.26
	± 0.08	± 0.16	± 0.15	± 0.05	± 0.15	± 0.19	± 0.36

N= number of tadpoles experimented per group

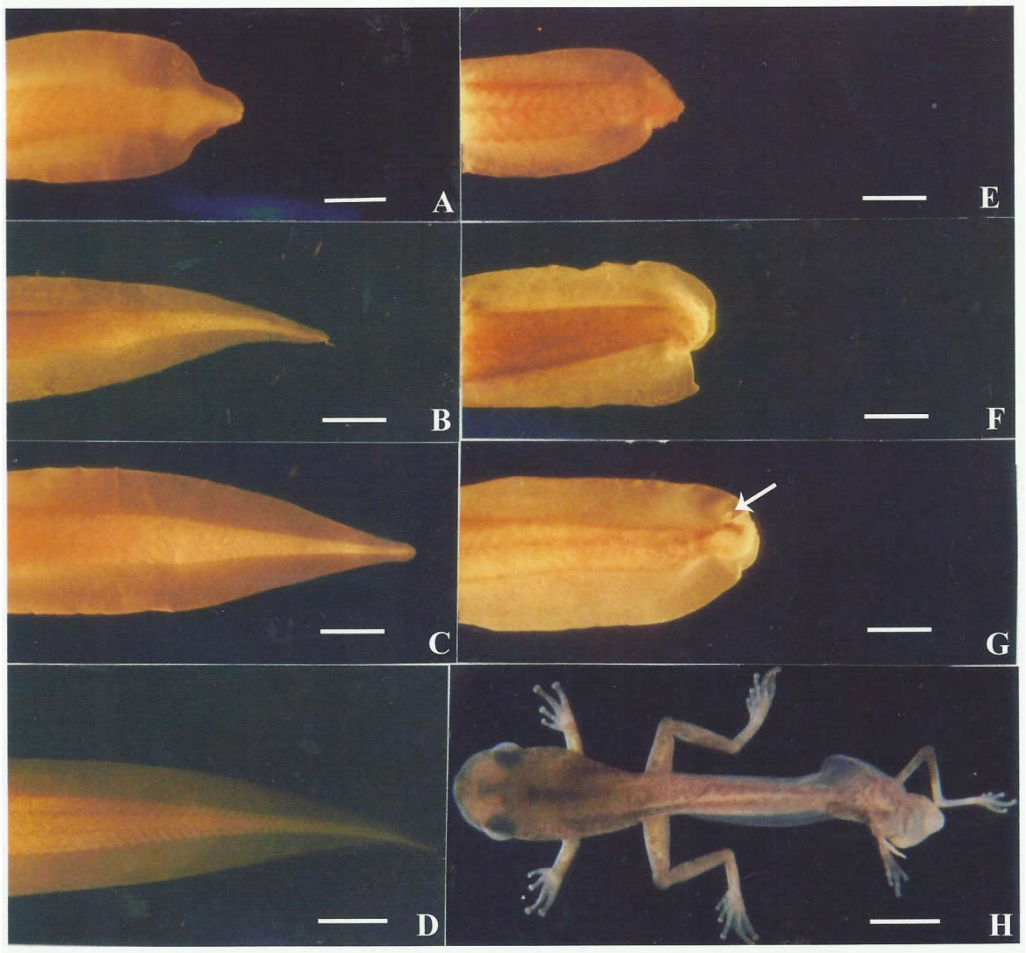

Fig. 1

Morphology of the original and regenerated tails of *Polypedates maculatus*. A. Regenerated tail of the control group 5 days after amputation. B. Tail of control group 10 days after amputation. C. Normal looking tail of the control group 15 days after amputation. D. Original tail before amputation. E. Abnormal tail regeneration with a blunt end 5 days after amputation F. Further enlargement of the abnormal tail 10 days post amputation G. A bulbular mass in 15 days post amputated tail with limb buds (arrows). H. A treated tadpole with ectopic limbs at the cut end of tail at the emergence of forelimbs (50 days post amputation). Scale bar: Fig. A to G=2mm; Fig. H=5mm)

was 7.43 ± 0.15 µmol pNP formed / mg protein / min. In the 10 days regenerated tails level of ALP increased to 10.56 ± 0.19 µmol pNP formed / mg protein / min. However, there was decline in the level of ALP in the 15 days regenerated tails and the level remained 9.26 ± 0.36 µmol pNP formed / mg protein / min.

DISCUSSION

Vitamin A led to abnormal tail regeneration in all the treated tadpoles and in 20% cases, ectopic limbs developed. Present study has shown an elevation in the level of specific activity of acid phosphatase (ACP) in the regenerating tails of the control group in comparison to the original tails. During tail regeneration in the control group, maximum level of ACP activity was observed in the 5 days regenerated tails. The level was 1.24 fold higher than the original tails. There was decline in the level of ACP in the 10 and 15 days regenerated tails. The level decreased by 1.05 and 1.16 fold in the 10 and 15 days regenerated tails, respectively than 5 day regenerated tails. However, the difference in the enzyme level was not significant between 5 and 10days regenerated tails of this group. In the treated group, significant rise in the level of ACP was observed in the abnormally regenerated tails as compared to the regenerated tails of the control group. In 5 days regenerated tails of the treated group there was 1.37 fold rise in the level of ACP than the original tails. The rise was 1.38 and 1.54 fold in the 10 and 15 days regenerated tails, respectively as compared to the original tails. In general the level of ACP

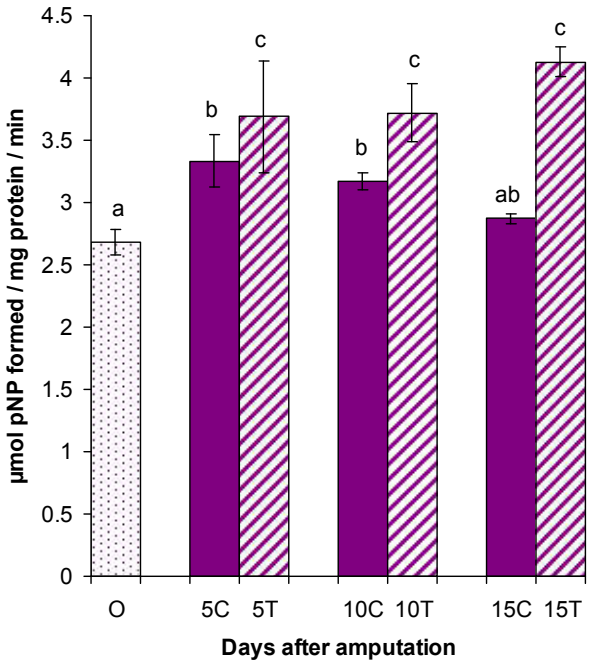

Figure 2. Specific activity of acid phosphatase in µmol p-nitrophenol (pNP) formed / mg protein / min at 37°C of the regenerated tails of vitamin A 10IU/ml treated (72h) and control tadpoles of *Polypedates maculatus.* O-Original, C-Control, T-Treated

remained higher in the regenerated tails of the treated group tadpoles than the controls and the maximum was observed in the 15days regenerated tails of the treated group. In the regenerated tails of both control and treated tadpoles the specific activity of alkaline phosphatase (ALP) remained higher than original tails (Figs. 3). In the control tadpoles the level increased by 3.76 fold in the 5 days regenerated tails as compared to original tails. There was further rise in the enzyme level (4.92 folds) in the 10 days regenerated tails. In the 15 days regenerated tails, the level declined by 1.71 fold than the 10 days regenerated tails. However, the level of ALP remained significantly higher in the regenerating tails of the control group than the original tails. In the treated group, level of ALP remained higher than the regenerated tails of the control group. There was 6.69, 9.51 and 8.34 fold rise in the level of ALP in the 5, 10 and 15 days regenerated tails as compared to the original tails, respectively. The maximum level was observed in 10 days regenerated tails.

Even though there was decline in the level of this enzyme in 15 day regenerated tails, the difference remained significantly higher in the treated group, than the respective regenerated tails of the control group. Niazi and Saxena (1979) have reported necrotic cells from tail regenerates of treated tadpoles. According to them, among the three axial tissues (muscle, notochord and spinal cord) of the tail regenerate, muscle was relatively more adversely affected and the notochord the least. They reported the nerve cord and the notochord to retain morphological identity at any time. Ju and Kim (1998) have shown degradation of extra cellular matrix (ECM) of muscle, nerve sheath and dermal tissue during limb regeneration in salamander. Degradation of ECM was accomplished by protease including acid hydrolyses such as cathepsin D and acid phosphatase (Ju and Kim, 1998), β glucuronidase and carboxylic ester hydrolases (Schmidt, 1966) as well as matrix metalloprotease (Yang *et al*., 1999). Acid hydrolases has been described to be released after amputation from injured and dying cells. The source of hydrolases were described to be wound epidermis, blastema stem cells themselves and macrophages (Stocum, 1995). Ju and Kim (1993, 1994) have reported pattern duplication by retinoic acid treatment in the regenerating limbs of the salamander, *Hynobius leechii* along with increase in acid phosphatase activity following retinoic acid treatment in the regenerating limbs. Lysosomal acid phosphatase is known to mediate differentiation in the regenerating salamander limb (Ju and Kim, 2010) Apoptosis or programmed cell death has been described to be required during the first 24 hours post amputation during tail regeneration in the tadpoles of *Xenopus laevis* (Tseng *et al*., 2007). When caspase-3 activity was inhibited, regeneration was abolished. Tseng *et al*., (2007) have interpreted these results and described existence of endogenous inhibitory cells. They have suggested death of these inhibitory cells (by programmed cell death or apoptosis) to be necessary for regeneration to occur. Present study has shown elevated level of specific activity of acid phosphatase during early stage of normal tail regeneration.

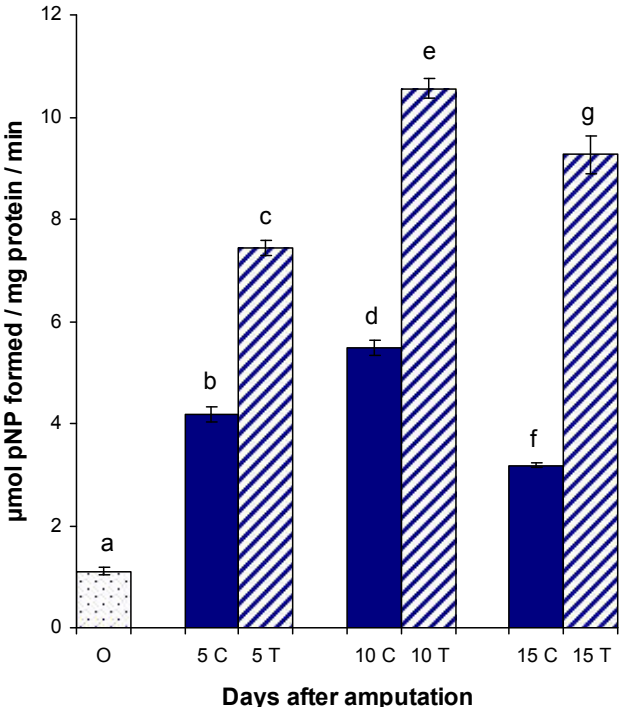

Figure 3. Specific activity of alkaline phosphatase in µmol p-nitrophenol (pNP) formed / mg protein / min at 37°C of the regenerated tails of vitamin A 10IU/ml treated (72h) and control tadpoles of *Polypedates maculatus.* O-Original, C-Control, T-Treated

In the regenerating tails of the treated tadpoles the activity of ACP remained comparatively higher than the regenerating tails of the control group, an indication of more lytic activities. A higher level of ACP in the abnormally regenerated tails following vitamin A treatment is suggested to be associated with cellular differentiation and subsequently leading to ectopic limb development as described by Ju and Kim (2010) during limb regeneration in salamander. In the regenerated tails of the present study the activity of alkaline phosphatase also remained higher than the original tails. In adult newt *Triturus pyrrohogaster,* alkaline phosphatase activity has been reported to remain high during limb regeneration (Inoue and Suzuki, 1969). In mouse tail epidermis synthesis of alkaline phosphatase stimulated by vitamin A has also been described (Riley and Spearman, 1968). A stronger alkaline phosphatase

activity has been evident in the regenerating tails than the controls in the tadpoles of *Bufo marinus* (Junqueira, 1950). Expression of alkaline phosphatase in blastemal cells of the regenerates has been described in newt during limb regeneration (Pecorino *et al*., 1996). Accumulation of undifferentiated blastemal cells below wound epidermis is evident during tail regeneration (Mochii *et al*., 2007). A comparatively large mass of undifferentiated cells accumulate in the tissue sections of vitamin A exposed abnormal tails (Mahapatra *et al*., 2004). Thus, higher activity of alkaline phosphatase in the abnormally regenerating tails of the treated tadpoles can be correlated with the presence of undifferentiated cells in the regenerated tails. It is evident from the present study that elevation in specific activity of acid and alkaline phosphatase occurs during normal tail regeneration. In the abnormally regenerated tails of the treated group, the activity of both the enzymes increases further. An elevated level of enzymes seems to be required for normal process of regeneration and further elevation in the level is associated with abnormal tail regeneration, a prerequisite for ectopic limb development.

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