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THE VARIOUS EFFECTS OF RETINOL PALMITATE ON HIND-LIMB REGENERATION IN *BUFO MELANOSTICTUS* TADPOLES OF DIFFERENT DEVELOPMENTAL STAGES

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INTRODUCTION

The unique effect of vitamin A and its derivatives (collectively called retinoids) on limb regeneration in amphibians is by now well documented. Contrary to the general rule of distal transformation of blastema, in regenerating amphibian limb, the blastema of retinoid treated anuran tadpoles and urodeles generally gives rise to a regenerate in which all or some of the structures are duplicated. Since the original discovery of this proximalizing effect of vitamin A in young toad tadpoles (Jangir and Niazi, 1978, Niazi and Saxena 1978); it has been confirmed in the tadpoles of several anuran species and the larvae and adults of several urodeles treated with various retinoids after limb amputation (*Anurans* : Maden, 1983; Niazi and Alam, 1984; Niazi and Ratnasamy, 1984; Niazi and Sharma, 1985; Scadding and Maden, 1986; *Urodeles* : Maden, 1982; Thoms and Stocum, 1984; Lheureux et al., 1986; Kim and Stocum, 1986; Koussoulakos et al; 1986). It is well known that in the anurans the capacity to regenerate declines progressively along the P-D axis of the limb, until it disappears throughout the limb some time before metamorphosis. The extent of this capacity varies at different P-D levels of the limbs during the various developmental stages of the tadpoles. In this communication we report the results of a study on the effects of retinol palmitate treatment on amputated limbs of *Bufo melanostictus* tadpoles, belonging to different stages. Amputations were performed through thigh, shank and ankle.

MATERIALS AND METHODS

The tadpoles belonging to stages 30/31, 34, 36 and 38 (Khan 1965) were used. At stage 30/31, the tiny hind-limb has

two constrictions marking the thigh-shank and shank-ankle junctions; the distal edge is either flattened or a small indentation marks the 5th toe rudiment. At stage 34 the limb is longer; thigh, shank and ankle+foot regions are distinct and shallow interdigital indentations distinguish the rudiments of all the five toes. At stage 36 the limb further elongates, the palmer region of the foot is closely applied to the lateral surface of the ventral tail fin. The anal tube extends up to the ankle. At stage 38 the limbs are well developed and functional, shank makes an acute angle with the thigh; tarsal region is parallel to the tail fin but the toes are directed away from the body axis; the anal tube disappears.

The tadpoles were narcotized in 1 : 4000 solution of MS222 (Sandoz) and then their limbs were amputated through thigh, shank or ankle region with a sharp blade under a stereoscopic binocular microscope. The control groups were reared in tap water for 15 days after amputation. The tadpoles of the experimental series were exposed to 15 IU/ml solution of retinol palmitate for 3 days (see Niazi and Ratnasamy, 1984) and then reared in tap water for the remaining 12 days. Each group consisted of 15-25 tadpoles. They were maximally fed with boiled spinach and the rearing medium was changed every other day. All experiments were carried out at room temperature (30-32°). On the 15th day the tadpoles were fixed in Bouin's fluid. The regenerates, in which post-blastemic redifferentiation had not occurred, were sectioned and stained with haematoxylin and eosin for histological study. The well formed regenerates were stained *in toto* with Victoria blue to visualise the skeletal structures.

RESULTS

The regenerates showed four types of structures in various proportions in different animal groups. (i) *Normal (N-type)* : these consisted of only the portion distal to amputation level (Fig. 1a). (ii) *Whole limb (WL-type)* : The regenerate was a complete limb proximo-distally containing a girdle, thigh, shank and ankle +foot structural elements, in this order. Such regenerates included cases in which one (Figs. 1b, c,d), two (Fig. 1e) and very occasionally three whole limbs developed from the same stump. (iii) *Persistent blastemas (PB-type)* : mostly one, and in a few cases two blastemas developed on the stump but they persisted as such

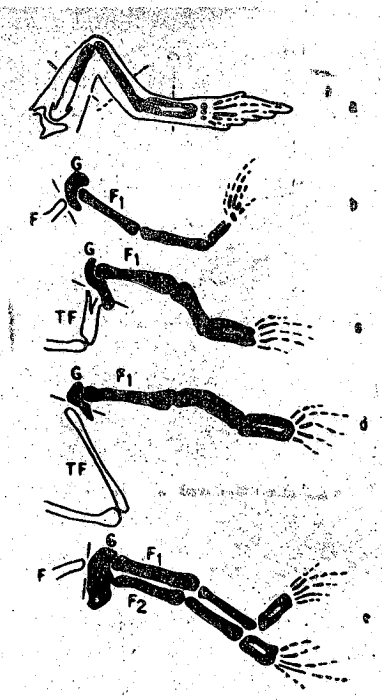

Fig. 1. Skeletal elements of 15 day old regenerated hind-limbs of *Bufo melanostictus* tadpoles, drawn from the photographs of Victoria blue preparations. Transverse lines (unlabelled) indicate the levels of amputation in each case (broken lines indicate the levels of amputations in other cases). (a) represents the control regenerate, (b—e) represent retinol palmitate treated regenerates.

F=stump femur; F_1, F_2=duplicate femurs; G=duplicate girdle TF=tibio-fibula of stump.

a. *N-type* regenerate of a control amputated through thigh. Regenerated portion consist of parts only distal to the level of amputation

b, c, d. Single *WL-type* regenerates developed after amputation through thigh (b), shank (c) and ankle (d) in treated tadpoles

e. Two mirror-imaged *WL-type* regenerates developed from the same stump after amputation through thigh in a treated tadpole of stage 30/31.

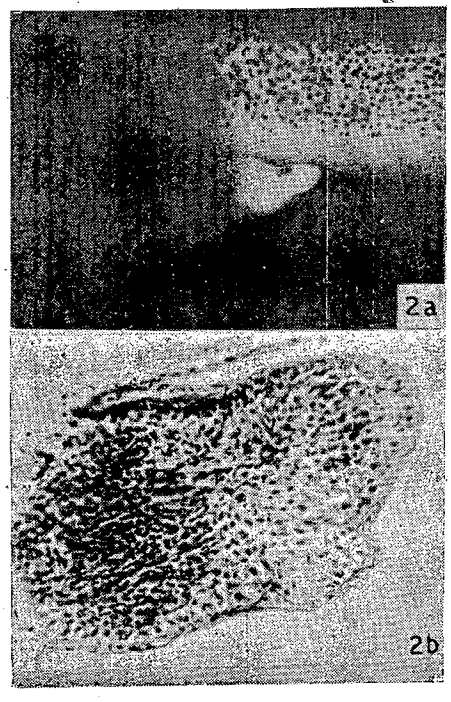

Fig. 2. 15 day old *PB-type* regenerate developed on a thigh stump of stage 30/31 tadpole. (a) external view; (b) L.S. of stump and regenerate.

without any morphogenesis or histogenesis (Fig. 2), (iv) Small spike-like out-growths contained some cartilage.

Each regenerate was examined for the number of digits in the foot and the average number of toes per *N-type* regenerate served as a measure of morphological quality. With very few exceptions, the *WL-type*, whether single or multiple, were pentadactylous.

Controls

Positive regenerative growth occurred in 100% cases from all the three levels of amputations up to stage 34. Thereafter, this tendency declined at all the levels until at stage 38, no regeneration of any kind occurred and only spikes developed in a few cases after amputation through ankle. At stage 30/31 the number of perfectly formed, pentadactylous regenerates was high.

In the older stages there was progressive increase in hypomorphism and oligodactyly indicated by the reduction in the number of toes in the thigh, shank and ankle level regenerates in this order (Table 1).

Treated tadpoles

The following features were noted :

(i) *WL-type* regeneration occurred only in stage 30/31 and 34 tadpoles. In the former all the well formed regenerates were of this type; the frequency of such regenerates was very high at both the thigh and shank levels but it was much lower at the ankle level. At stage 34, both *N-type* and *WL-type* regenerates were produced but in significantly different proportions at all the 3 levels (Table 1).

(ii) The *WL-type* regenerates included both single as well as multiple ones arising from the same stump. The multiple types constituted about 1/3 to 1/2 of all the *WL* regenerates but this propensity was greater in older tadpoles.

(iii) Almost all regenerates from the three levels at stage 36 were of *N-type* and well developed. Also, the *N-type* regenerates of stage 34 treated tadpoles were structurally better formed and had more digits as compared to the corresponding controls (table 1).

(iv) Whereas, the controls possessed no regenerative capacity at stage 38 in thigh and shank level amputations, the treated blastemas showed small spikes with a little cartilage in 10 to 20% cases. At ankle level amputations, however, only a few controls had spike formation, while the treated blastemas formed spikes in 50% cases ((Table 1).

(v) The incidence of inhibited regenerates (*PB-type*) varied significantly at various levels of amputations in the tadpoles of different stages but no such case was found at any level in stage 38 tadpoles (Table 1).

DISCUSSION

The results demonstrate that even an identical treatment with retinol palmitate produces different effects on limb regeneration.

TABLE 1

Effect of retinol palmitate on hind-limb regeneration in *Bufo melanostictus* tadpoles of different developmental stages

Stage	Amputation level	Group	% amputated limbs showing regeneration	% of the three types of regenerates			% pentadactylous limbs in *N-type* regenerates (average no. of toes/regenerate)
				N	*WL*	*PB*	
30/31	Thigh	Control	100	100	—	—	84 (4.7)
		Treated	100	—	90	10	—
	Shank	Control	100	100	—	—	87 (4.8)
		Treated	100	—	95	5	—
	Ankle	Control	100	100	—	—	85 (4.8)
		Treated	100	—	56	44	—
34	Thigh	Control	100	100	—	—	40 (3.9)
		Treated	100	36	16	48	67 (4.7)
	Shank	Control	100	100	—	—	47 (4.1)
		Treated	100	12	60	28	100 (5.0)
	Ankle	Control	100	100	—	—	67 (4.6)
		Treated	100	8	76	24	100 (5.0)
36	Thigh	Control	43	100	—	—	0.0 (1.8)
		Treated	52	53	—	47	14 (3.8)
	Shank	Control	67	100	—	—	30 (3.5)
		Treated	72	100	—	—	50 (4.8)
	Ankle	Control	73	100	—	—	27 (4.0)
		Treated	85	100	—	—	59 (4.6)
38	Thigh	Control	0	—	—	—	—
		Treated	10*	—	—	—	—
	Shank	Control	0	—	—	—	—
		Treated	20*	—	—	—	—
	Ankle	Control	15*	—	—	—	—
		Treated	50*	—	—	—	—

*Small spikes

N, *WL* and *PB*; see text for explanation.

The frequency and quantum of regeneration depends on the level of amputation and the stage of development of the tadpoles. Nearly similar observations have been made on *Rana breviceps* tadpoles (Niazi and Sharma, 1985; Sharma and Niazi, 1986). The present results further show that the proximalizing effect of retinoid treatment is correlated with the presence of high ability to regenerate normally. The *WL-type* regenerates were obtained only at stages

30/31 and 34 when the power to regenerate was high at all the 3 levels. However, it should be noted that whereas the regenerates of stage 30/31 controls were perfect in 84-87% cases, the number of such good pentadactylous regenerates at stage 34 was much less. As indicated by the percentage of pentadactylous regenerates of the controls at this stage and the average number of toes per regenerate, the regeneration ability at the 3 P-D levels was thigh < shank < ankle. It is significant that the frequency of proximalized *WL-type* regenerates obtained at the 3 levels in stage 34 treated tadpoles showed a P-D gradient similar to that of the perfect regenerates of the control series of this stage, i.e. thigh < shank < ankle (see Table 1). With further reduction of the normal power of regeneration at all levels at stage 36, retinoid treatment failed to produce a single case of proximalized regenerate. This is indicated by increased oligodactyly of the regenerates and complete absence of regeneration at stage 38. The observations of Scadding and Maden (1986) on *Xenopus* and of Sharma and Niazi (1986) on older tadpoles of *Rana breviceps* together with the present results on *B. melanostictus* provide strong support for the suggestion that the capacity of retinoids to proximalize limb regeneration in anurans is correlated with their normal ability to regenerate. The urodeles commonly used in such studies are all able to regenerate limbs throughout life, and in them production or duplication of stump structures in the regenerates has been achieved by retinoid treatment in both larvae and adults. The correlation of this effect of retinoids with the power of regeneration may be the general rule for all amphibians.

In the treated groups, a number of good regenerates at stage 34 and all of them at stage 36 were of *N-type*, in which the retinoid had failed to produce duplication of any stump element. However, these were better formed morphologically than those of the corresponding controls. Retinoids cause extensive dissolution of stump tissues, liberating large number of cells resembling dedifferentiated mesenchymal cells, and also delay the onset of redifferentiation of the blastema (Jangir and Niazi, 1978; Kim and Stocum, 1986; Lheureux et al., 1986; Sharma and Anton, 1986); Vitamin A treatment also reduces, or suppresses, thyroid activity in the tadpoles, delaying or preventing metamorphosis (Sharma and Niazi, 1983). It is known that increasing thyroid activity expedites redifferentiation. It is suggested that these effects may

be involved in the improved regeneration in cases where the retinoid is unable to cause proximalization of the regenerates. The induction of regeneration, even though of a very low order, in the non-regenerating limbs may also be ascribed to the same effects.

Exposure of regenerating limbs to retinoids beyond blastema stage has been found to inhibit morphogenesis and redifferentiation of the blastema (Saxena and Niazi, 1977; Jangir and Niazi, 1978). During the present study, however, a fairly large number of inhibited *PB-type* regenerates were obtained even though the treatment was restricted to the period of blastema formation (3 days post - amputation). Perhaps, within the same group some individuals may have ingested more of the drug from the rearing medium than others. However, this assumption does not fully explain the pattern of variations in the frequency of such regenerates at various levels in tadpoles of different stages. Possibly, these variations may also be due to differences in the stage of differentiation of tissues at the three P-D levels of the developing limb, at different stages of development of the tadpoles. The blastema cells emerging from the tissues at different levels of histogenesis may differ in their sensitivity to the same retinoid treatment.

Frequent production of more than one proximalized regenerates from the same stump in the treated tadpoles, also observed in *Pleurodeles* larvae (Lheureux et al., 1986), is another striking feature. The reasons for this need to be looked for in the initial stages of regeneration and beginning of the treatment. Formation of twin blastemas on the same stump was observed within 2-3 days by Saxena and Niazi (1977) in the toad tadpoles exposed to vitamin A treatment after amputation of the limbs.

REFERENCES

Jangir OP and Niazi IA (1978) Stage dependent effects of vitamin A excess on limbs during ontogenesis and regeneration in tadpoles of the toad *Bufo melanostictus* (Schneider). Indian Journal of Experimental Biology 16 : 438-445

Khan MS (1965) A Normal Table of *Bufo melanostictus* (Schneider). Biologia 11 : 1-39

Kim WS and Stocum DL (1986) Effects of retinoic acid on regenerating normal and double half limbs of axolotls-histological studies. Roux' Archives Developmental Biology 195 : 243-251

Koussoulakos S, Sharma KK and Anton HJ (1986) Effect of vitamin A palmitate on the growth of regenerate and blastema DNA content of *Triturus alpestris*, Hell. Biochem. Biophys. Society Newsletter 23 : 14-15

Lheureux E, Thoms SD and Cary F (1986) The effects of two retinoids on limb regeneration in *Pleurodeles waltl* and *Triturus vulgaris*. Journal of Embryology and Experimental Morphology 92 : 165-182

Maden M (1982) Vitamin A and pattern formation in the regenerating limb. Nature (London) 295 : 672-675

Maden M (1983) The effect of vitamin A on limb regeneration in *Rana temporaria*. Developmental Biology 98 : 409-416

Niazi IA and Alam S (1984) Regeneration of whole limbs from shank stumps in toad tadpoles treated with vitamin A. Roux' Archives Developmental Biology 193 : 111-116

Niazi, IA and Ratnasamy, CS (1984) Regeneration of whole limbs in toad tadpoles treated with retinol palmitate after the wound healing stage. Journal of Experimental Zoology 230 : 501-550

Niazi, IA and Saxena, S (1978) Abnormal hindlimb regeneration in tadpoles of the toad, *Bufo andersonii*, exposed to excess vitamin A. Folia Biologica (Krakow), 26 : 3-11

Niazi, IA and Sharma, KK (1985) Variations in the pattern of regeneration from different proximo-distal levels of limbs in young frog tadpoles treated with retinol palmitate. Cell Differentiation *16* (Suppl.) : 16 S Abstract).

Saxena, S and Niazi, IA (1977) Effect of vitamin A excess on hindlimb regeneration in tadpoles of the toad, *Bufo andersonii* Boulenger. Indian Journal of Experimental Biology *15* : 435-439

Scadding SR and Maden, M (1986) Comparison of the effects of vitamin A on limb development and regeneration in *Xenopus laevis* tadpoles. Journal of Embryology and Experimental Morphology *91* : 35-53

Sharma, KK and Anton, HJ (1986) Biochemical and ultrastructural studies on vitamin A induced proximalization of limb regeneration in axolotl. *In* "Progress in Developmental Biology", Part A, 105-108 (Alan R Liss Inc.)

Sharma, KK and Niazi, IA (1983) Effect of vitamin A on metamorphosis in tadpoles of *Rana breviceps*. National Academy of Sciences Letters *6* : 397-399

Sharma, KK and Niazi, IA (1986) Variety of regenerative responses of different proximo - distal limb segments of young and advanced *Rana breviceps* tadpoles treated wiih vitamin A palmitate after amputation. *In* : "Monographs in Developmental Biology" (ed. HJ Anton) Karger, Basel (in press)

Thoms, SD and Stocum, DL (1984) Retinoic acid induced pattern duplication in regenerating urodele limbs. Developmental Biology 103 : 319-328