THE JOURNAL OF EXPERIMENTAL ZOOLOGY 230: 501–505 (1984)

RAPID COMMUNICATION

Regeneration of Whole Limbs in Toad Tadpoles Treated With Retinol Palmitate After the Wound-Healing Stage

I.A. NIAZI AND C.S. RATNASAMY
Developmental Biology Laboratory, Department of Zoology, University of Rajasthan, Jaipur 302004, India

ABSTRACT In young *Bufo melanosticus* tadpoles at the spatula stage of limb development, the left hindlimb was amputated through the shank and the right hindlimb was left intact. One day later, when the amputation surface was covered by wound epidermis, the tadpoles were immersed in 15 IU/ml of retinol palmitate solution for 2 days up to blastema formation and thereafter reared in plain water. Contrary to the situation in untreated controls, all the resultant regenerates of the vitamin-treated tadpoles were complete limbs containing elements from the pelvic girdle to the phalanges. Retinol palmitate treatment inhibited the development of the foot in the unamputated right limbs. These results show that retinol palmitate has a different effect on dedifferentiating cells of a regenerating limb from that on cells that are differentiating in a normally developing limb.

Since its discovery more than 200 years ago, numerous investigators have studied the phenomenon of limb regeneration in amphibians. They have observed that what is regenerated, through the formation of a regeneration blastema, is never more than the parts distal to the level at which the limb is amputated, a fact known as the "rule of distal transformation" (Rose,'62). Until recently, none of the variety of procedures employed in research on this phenomenon had resulted in the development of regenerates undisputably containing any structure belonging to limb regions proximal to the amputation plane (Wallace, '81; Stocum, '84). The many compounds tested for their effects on regeneration were found to either inhibit it, or merely increase the growth rate of the regenerates to some extent (Wallace, '81). However, vitamin A has recently been found to have a unique effect on regeneration. Niazi and Saxena ('78) discovered that immersion of the tadpoles of the toad *Bufo andersonii* in retinol palmitate after amputation of the hindlimb through the shank resulted in several regenerates consisting of all limb segments, including the thigh, shank, ankle, and foot, not merely the parts distal to the amputation level. Since then, this effect of vitamin A and its derivatives on limb regeneration has been confirmed in several other amphibian species, including the anurans *Bufo melanostictus* (Jangir and Niazi, '78; Alam, '83; Niazi and Alam, '84), *Rana breviceps* (Sharma, '82) and *R. temporaria* (Maden, '83a), the urodeles *Ambystoma mexicanum* (Maden, '82; '83b), and the adult newt *Notophthalmus viridescens* (Thoms and Stocum, '84). In the case of the anuran tadpoles, multiple limbs, usually mirror-image twins, frequently regenerate from the same stump (Jangir and Niazi. '78; Sharma, '82; Alam, '83; Niazi, '83; Maden, '83a); in urodeles, extra fingers and an occasional extra zeugopodial bone have been encountered, mostly in adult newts (Thoms and Stocum, '84).

The specific effect of vitamin A depends on the stage of regeneration at which it is administered. In anuran tadpoles, regeneration is inhibited if the tadpoles are maintained in the vitamin solution beyond the blastema stage (Saxena and Niazi, '77; Sharma, '82; Alam, '83) or when the treatment is started

Address reprint requests to Dr. I.A. Niazi at his current address: Department of Genetics and Development, University of Illinois, 515 Morrill Hall, 505 S. Goodwin Ave., Urbana, IL 61801, USA.

© 1984 ALAN R. LISS, INC.

after the blastema is formed (Jangir and Niazi, '78; Niazi, '83). These observations indicate that the positive effect of vitamin A is exerted during the early stages of the regenerative process up to blastema formation.

The objective of the present investigation was to explore whether it is necessary for vitamin A to act on all the early phases of regeneration to cause the appearance in the regenerate of structures proximal to the amputation level. Specifically, must the vitamin be present during the wound-healing phase, or is its presence during dedifferentiation and blastema cell accumulation sufficient? The results show that whole-limb regenerates can be obtained by treatment with retinol palmitate after wound healing has occurred.

MATERIALS AND METHODS

Tadpoles of the toad, *Bufo melanostictus* Schneider, were used in this study. At the start of the experiment they were at stage 30/31 according to the normal table for this species (Khan, '65). At this stage the stylopodial (thigh) and zeugopodial (shank) segments of the hindlimbs are demarcated from each other by a distinct knee bend, and the autopodial segment (ankle and foot) is spatula shaped. The tadpoles were narcotized in a 1:4,000 solution of MS-222 (Sandoz) and the left limb was amputated under a stereomicroscope with a sharp razor blade below the knee through the shank. The right limb of the experimental animals was not amputated. Vitamin A is known to inhibit morphogenesis of the foot when administered at the spatulate stage (Jangir and Niazi, '78). Hence, in this experiment its effect on foot morphogenesis at a slightly later stage could be determined.

It was established by a preliminary study that within 24 h postamputation, the cut end of the shank stump is covered by a two-layered wound epithelium, and the region below it becomes free from dead cells and tissue fragments. Dedifferentiation of stump cells and blastema formation takes place over the next 2 days, and redifferentiation begins on the fourth day postamputation. The tadpoles were therefore kept in tap water for the first 24 h postamputation and were then immersed in a 15 IU/ml solution of vitamin A palmitate for the next two days. In our laboratory, this concentration has proved the most effective in inducing a proximalizing effect on regeneration of toad tadpole hindlimbs. The vitamin solution was prepared by dissolving a known quantity of an oily solution of vitamin A palmitate (Arovit, Roche, India) in a few drops of ethanol and diluting it with tap water to obtain the desired concentration. The tadpoles were then reared in tap water for another 12 days. Control tadpoles amputated through the left shank were reared in tap water throughout the 15 days.

The control and experimental groups each consisted of 20 tadpoles. They were maintained at room temperature (30–32°C) in enamel bowls. The rearing medium was changed every day and the tadpoles were fed maximally with freshly boiled spinach leaves. There was no mortality. On day 15 the animals were fixed in Bouin's solution; the unamputated and regenerated limbs were photographed and then stained with Victoria Blue B for study of their skeletal organization.

RESULTS

Well-formed regenerates developed in all control and treated tadpoles. As expected, control regenerates consisted of parts distal to the amputation level (Fig. 1) The proximodistal organization of the treated regenerates, however, was quite different. First, although the shank stump grew in length somewhat during the 15 days of the experiment, the removed portion of the shank was not restored. Instead, in 20 out of 20 cases, the regenerates consisted of whole limbs consisting of a partially or completely formed pelvic girdle, stylopodium (femur), zeugopodium (tibia-fibula), and autopodium (tarsals, metatarsals, and phalanges) in proper proximodistal sequence (Figs. 2–5). The skeleton of these regenerates was not connected to that of the shank stump (Fig. 5). In two of these cases, a supernumerary regenerate arose from the amputation site along with the primary regenerate (Fig. 3). Like the primaries, the supernumeraries each contained a complete limb skeleton, the girdle of which was fused with that of the primary (Fig. 5). The primaries and supernumeraries were encased in common skin in the thigh region (Fig. 3).

Morphogenesis of the autopodium of the regenerates was normal with well-developed tarsals, metatarsals, and phalanges. In most cases the feet possessed the full complement of five toes (Figs. 2–5). The development of the autopodium in the contralateral, unamputated limb, however, was very defective in all 20 cases. In no case did there develop more than one metatarsal and one toe (the fourth), and even this contained fewer than the usual number of phalanges (Figs. 2, 6). Development and growth of the unoperated right limbs of all control tadpoles were normal in all respects.

DISCUSSION

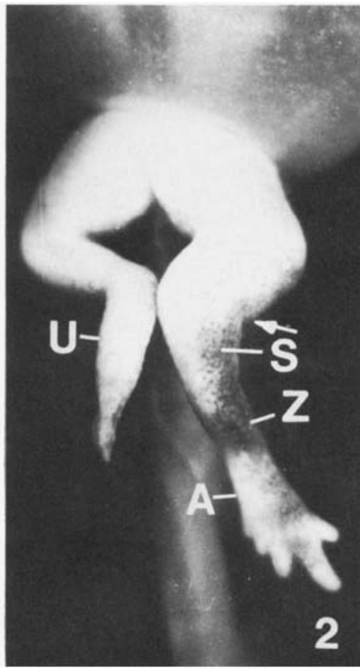
The present study shows first that it is not necessary that vitamin A palmitate be present during the wound-healing phase of hindlimb regeneration in *Bufo melanostictus* tadpoles to effect proximalization. Administration of 15 IU/ml of the vitamin during the stages of dedifferentiation and blastema formation was sufficient to cause regeneration of whole limbs from the shank stump in 100% of the cases. Since other studies have shown that exposure of anuran tadpoles to vitamin A after blastema formation inhibits regeneration (Jangir and Niazi, '78; Niazi, '83), the data suggest that the stages of dedifferentiation and blastema formation are particularly susceptible to the unique proximalizing effect of the vitamin.

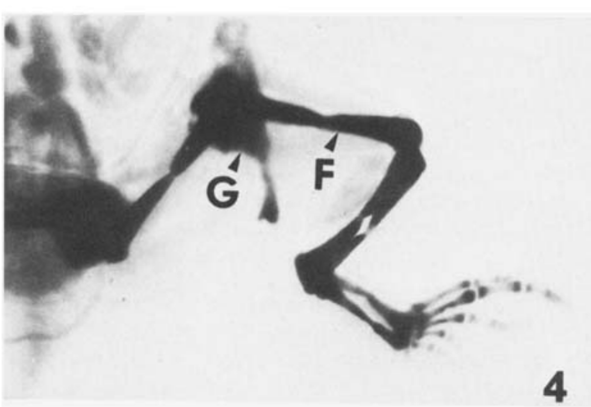
The nature of the vitamin A effect on these stages is unknown. One possibility is that vitamin A may enhance the dedifferentiative process. Studies on avian and mammalian tissues and cells have shown that vitamin A causes dissolution of bone and cartilage matrix by increasing synthesis and release of lysosomal hydrolases, resulting in the liberation of healthy cells that are mesenchymal in appearance, and has also been shown to promote mitosis in various cell types (Dingle et al., '72; Fell and Rinaldini, '65; Ganguly et al., '80). Enhanced acid phosphatase activity (Sharma, '82) and increased mitosis (Alam, '83) have been observed in the regenerating limbs of frog and toad tadpoles treated with vitamin A. However, it has recently been shown in regenerating urodele limbs that, although vitamin A treatment causes loss of metachromasia from cartilage matrix, there does not seem to be an excess of dedifferentiated cells (Maden, '83b). Hence, the enhancement of morphogenetic potential by vitamin A is most likely due to a more profound mechanism than just intensification of tissue dissolution and enhanced mitosis.

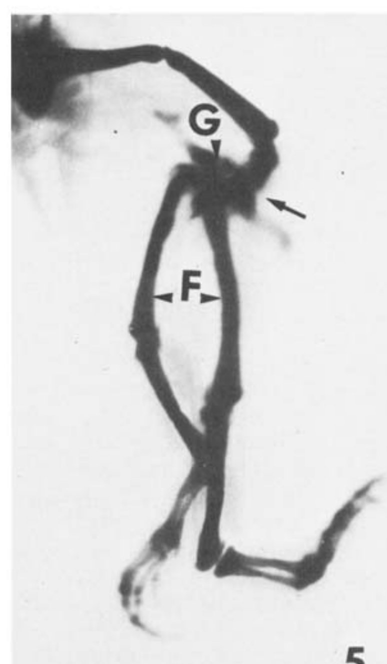
Based on the theory of positional information (Wolpert, '69), it is assumed that cells at every level along the proximodistal axis of the limb carry a memory of their position on this axis which limits the capacity of the regeneration blastema to the formation only of structures distal to the amputation plane (Pescitelli and Stocum, '80). It has been postulated that this memory resides in the cell surface coat and is reflected in the adhesive properties of blastema cells derived from different limb levels (Nardi and Stocum, '83). The specific molecular basis of this memory is unknown, but may involve the glycoproteins of the surface coat (Stocum, '84). In any case, vitamin A erases the positional memory, no matter how or where it may be encoded in limb cells, so that in addition to distal structures the blastema can also form structures proximal to the level of amputation.

The second thing shown in the present study is that vitamin A inhibits morphogenesis of the autopodium in developing limbs when given at a stage where the rudiment of one toe had developed. The differentiating tarsals and metatarsals were not apparent; the only part of the foot to develop was the fourth toe, and even this toe was deficient in phalanges. These results are in conformity with the finding that in anuran tadpoles (Jangir and Niazi, '78), as in mouse fetuses (Kochhar, '73; Nakamura, '77), the early developmental stages of the limb prior to the start of morphogenesis or histogenesis are susceptible to a destructive action of vitamin A. The specific mechanism of this action is unknown.

It is clear that vitamin A has different effects on developing and regenerating limbs, and on regenerating limbs it has different stage-specific effects. At present, we do not know the mechanistic details of these different effects. However, the basic difference appears to lie in the way vitamin A affects differentiating and dedifferentiating cells. This may indicate some intrinsic difference between these cell states, or a difference imposed by different environmental conditions under which these two cell states are found. For example, one difference between the developing, unamputated limbs and the regenerating limbs at the time of vitamin A administration in this study was the presence of a wound epidermis with an apical cap in the latter while normal epidermis was present in the former. Wound epidermis and the apical cap have some properties different from those of normal epidermis. They are involved in initiating dedifferentiation and maintaining blastema cells in an undifferentiated condition (Tassava and Mescher,







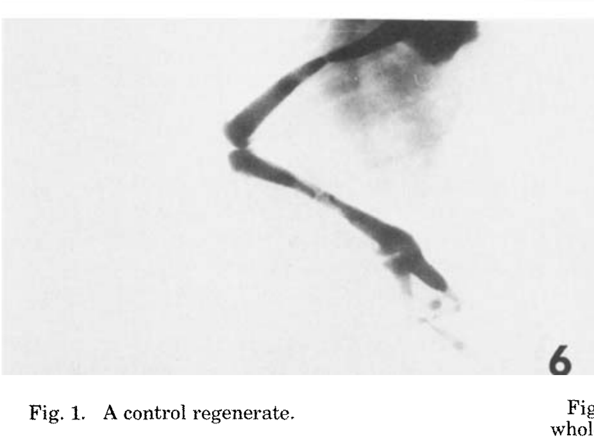


Fig. 1. A control regenerate.

Figs. 2, 3. A single (Fig. 2) and a case of twin (Fig. 3) whole limbs regenerated from shank stumps in tadpoles treated with retinol palmitate during days 2 and 3 postamputation.

Figs. 4, 5. Victoria Blue–stained skeletons of a single whole-limb regenerate (Fig. 4) and of the twin regenerates (Fig. 5) shown in Figure 3.

Fig. 6. Victoria Blue–stained skeleton of the unoperated right limb of a treated tadpole. Note that morphogenesis of the foot was inhibited.

Abbreviations

Arrow, amputation level.
A, autopodium;
F, Femur;
G, girdle;
S, stylopodium, and
Z, zeugopodium of regenerate.
U, foot of the unoperated limb.
All magnifications, 5×.

'75). In addition, the mesoderm of the amputated limbs was injured prior to vitamin A treatment, whereas that of the unamputated limbs was not, and the injury effect is over once the phase of blastema formation is past. It may be that amputational injury and the effects of the subsequently formed wound epidermis may have some role in preventing the action of vitamin A on dedifferentiating cells from becoming lethal and at the same time permitting the acquisition by these cells of greater than normal morphogenetic capability. Whether the proximalizing effect seen in the regenerating limbs is due primarily to effects of the vitamin on the epidermis or the mesenchyme, or both, is currently under investigation.

ACKNOWLEDGMENTS

The authors are grateful to Professor D.L. Stocum for his critical advice and suggestions in the preparation of the manuscript. This study was supported by grant 23-1231/80 (SR-II) from the University Grants Commission of India.

LITERATURE CITED

Alam, S. (1983) Studies on the morphogenetic influence of treatment of tadpoles of the anuran *Bufo melanostictus* (Schneider) with vitamin A palmitate on limb regeneration. Ph.D. thesis, University of Rajasthan, Jaipur, India.

Dingle, J.T., H.B. Fell, and D.S. Goodman (1972) The effect of retinol and of retinol binding protein on embryonic skeletal tissue in organ culture. J. Cell Sci., *11:*393–402.

Fell, H.B., and L.M. Rinaldini (1965) The effects of vitamins A and C on cells and tissues in culture. In: Cells and Tissues in Culture. E.N. Willmer, ed. Academic Press, New York and London, Vol. I, pp. 659–699.

Ganguly, J., M.R.S. Rao, S.K. Murthy, and K. Sarda (1980) Systemic mode of action of vitamin A. Vitam. Horm. *38:*1–54.

Jangir, O.P., and I.A. Niazi (1978) Stage dependent effects of vitamin A excess on limbs during ontogenesis and regeneration in tadpoles of the toad *Bufo melanostictus* (Schneider). Indian J. Exp. Biol., *16:* 438–445.

Khan, M.S. (1965) A normal table of *Bufo melanostictus* (Schneider). Biologia, *11:*1–39.

Kochhar, D.M. (1973) Limb development in mouse embryos. Analysis of teratogenic effects of retinoic acid. Teratology, *7:*289–298.

Maden, M. (1982) Vitamin A and pattern formation in the regenerating limb. Nature (Lond.), *295:*672–675.

Maden, M. (1983a) The effect of vitamin A on limb regeneration in *Rana temporaria.* Dev. Biol., *98:*409–416.

Maden, M. (1983b) The effect of vitamin A on the regenerating axolotl limb. J. Embryol. Exp. Morphol., *77:*273–295.

Nakamura, H. (1977) Digital anomalies in the embryonic mouse limb cultured in the presence of excess vitamin A. Teratology, *16:*195–202.

Nardi, J.B., and D.L. Stocum (1983) Surface properties of regenerating limb cells: Evidence for gradation along the proximodistal axis. Differentiation, *25:*27–31.

Niazi, I.A. (1983) Regeneration studies in India. In: Developmental Biology: An Afro-Asian Perspective. S.C. Goel and R. Bellairs, eds. Indian Society of Developmental Biology, Pune, India, pp. 161–176.

Niazi, I.A., and S. Alam (1984) Regeneration of whole limbs from shank stumps in toad tadpoles treated with vitamin A. Roux's Arch. Dev. Biol, *193:*111–116.

Niazi, I.A., and S. Saxena (1978) Abnormal hindlimb regeneration in tadpoles of the toad, *Bufo andersonii*, exposed to excess vitamin A. Folia Biol. (Krakow), *26:*3–8.

Pescitelli, M.J. Jr., and D.L. Stocum (1980) The origin of skeletal structures during intercalary regeneration of larval *Ambystoma* limbs. Dev. Biol., *79:*255–275.

Rose, S.M. (1962) Tissue-arc control of regeneration in the amphibian limb. In: Regeneration. D. Rudnick, ed. Ronald Press, New York, pp. 153–176.

Saxena, S., and I.A. Niazi (1977) Effect of vitamin A excess on hind limb regeneration in tadpoles of the toad, *Bufo andersonii* (Boulenger). Indian J. Exp. Biol., *15:*435–439.

Sharma, K.K. (1982) Investigations on limb regeneration in tadpoles and froglets of the anuran *Rana breviceps* (Schneider) treated with vitamin A or electrically stimulated. Ph.D. thesis, University of Rajasthan, Jaipur, India.

Stocum, D.L. (1984) The urodele limb regeneration blastema: Determination and organization of the morphogenetic field. Differentiation (in press).

Tassava, R.A., and A.L. Mescher (1975) The roles of injury, nerves and the wound epidermis during the initiation of amphibian limb regeneration. Differentiation, *4:*23–24.

Thoms, S.D., and D.L. Stocum (1984) Retinoic acid-induced pattern duplication in regenerating urodele limbs. Dev. Biol. (in press).

Wallace, H. (1981) Vertebrate Limb Regeneration. John Wiley, Chichester, New York, Brisbane, and Toronto.

Wolpert, L. (1969) Positional information and spatial pattern of cellular differentiation. J. Theor. Biol., *25:*1–47.