Effects of retinoic acid on regenerating normal and double half limbs of axolotls

Histological studies

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Summary. Retinoids induce proximodistal (PD) pattern duplication in zeugopodial (lower arm or leg) regenerates of normal limbs and PD pattern duplication plus anteroposterior (AP) pattern completion in double anterior half zeugopodial regenerates. In contrast, retinoids inhibit the regeneration of double posterior half zeugopodia (Kim and Stocum, 1986). Here we describe the developmental histology of regenerating normal, double anterior half and double posterior half zeugopodia in axolotls after intraperitoneal injection of retinoic acid (RA) at the stage of initial blastema cell accumulation. In all three classes of RA-treated limbs, the accumulation of blastema cells disappeared within 3 days after injection, and dedifferentiation continued to a much more proximal extent than in controls. Subsequently, however, the developmental histology of the three limb classes was different. RA-treated double posterior limbs exhibited the histological features typical of non-regenerating limbs: the premature appearance of a thick basement membrane under the wound epidermis, formation of a thick connective tissue mat between the basement membrane and the cut ends of the stump cartilages, and failure of blastema formation. In contrast, RA-treated normal zeugopodia formed single blastemas which grew out in a posterior or posterodorsal direction. RA-treated double anterior zeugopodia formed twin blastemas that were spatially separated to varying degrees and which grew distally. The blastemas of both these RA-treated limb types consisted of a proximal, low-density cell population that formed the girdle of the regenerate and a distal, high-density cell population that formed the free limb. In the free limb portion of the blastema, the density of the mesenchymal cell population was higher than in controls. Blastemas of RA-treated normal and double anterior zeugopodia appeared similar in size and proportions to controls at the medium bud stage, but subsequently took on the characteristics of stylopodial blastemas. These observations suggest that the extra pattern induced by RA in regenerating urodele limbs may be correlated with an increase in the number of de-differentiated cells per unit of blastema volume.

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Introduction

Vitamin A and its derivatives (retinoids) have been shown to cause anteroposterior (AP) pattern duplication in embryonic chick limb buds (Tickle et al. 1982; Summerbell 1983) and regenerating anuran hindlimb buds (Niazi and Saxena 1978; Maden 1983b), and to cause proximodistal (PD) duplication of stump structures in regenerating anuran hindlimb buds and mature limbs of urodeles (Niazi and Saxena 1978; Niazi et al. 1985; Maden 1982, 1983a; Thoms and Stocum 1984).

In contrast to the PD duplication observed after simple amputation of mature limbs in urodeles, retinoids induce pattern completion after removal of the posterior half of the limb prior to amputation (Stocum and Thoms 1984; Kim and Stocum 1986). Anterior half zeugopodia regenerate PD duplicated limbs with normal AP asymmetry. Double anterior half zeugopodia regenerate PD-duplicated, mirror-imaged regenerates with a complete and normal AP pattern, the right and left limbs arising from the corresponding right and left anterior half stumps. Posterior and double posterior half zeugopodia, however, fail to regenerate. These results have been interpreted to mean that retinoids proximalize positional memory in the PD axis and posteriorize it in the AP axis (Kim and Stocum 1986).

Maden (1983a) has reported the only histological observations on retinoid-treated normal limbs, and no histological studies of retinoid-treated half or double half limbs are available. Such studies are important not only for what they reveal about the tissue organization of retinoid-treated regenerating limbs, but for determining what cellular and subcellular aspects to focus on in analyzing the effects of retinoids on pattern regulation. Therefore we have surveyed the developmental histology of normal, double anterior half and double posterior half limb regenerates of axolotls treated with retinoic acid.

Materials and methods

Animals and maintenance. Larval axolotls (Ambystoma mexicanum) were provided by the Indiana University axolotl colony or obtained from spawns of our laboratory stock. They were reared individually in waxed paper cups containing 50% Holtfreter solution at room temperature (21°C),
and fed freshly hatched brine shrimp or frozen brine shrimp every other day. At the time of retinoic acid injection, the average weight of the animals was 0.7 grams in the series of experiments on normal limbs and 2.1 grams in the series of experiments on double half limbs.

**Operations.** Normal forelimbs were amputated bilaterally at the level of the distal zeugopodium or stylopodium (Fig. 1A). Figure 1B diagrams the surgical procedures used to construct double half limbs. Double anterior and double posterior half forelimb zeugopodia were constructed by (1) making a midline cut through the carpals between digits 2 and 3, and between the radius and ulna, (2) making a transverse cut at the elbow to remove the anterior half from the left limb and the posterior half from the right limb, and (3) exchanging anterior and posterior halves between the contralateral limbs to make a double anterior half limb in the right zeugopodium and a double posterior half limb in the left zeugopodium. Any protruding donor cartilage was trimmed for a good fit to the host limb.

The grafts were allowed to heal for 7 days and special care was taken during this period when changing water after feeding. The limbs were then amputated at the level of the distal zeugopodium, and protruding cartilages were trimmed to make a flat amputation surface. Blood circulation was observed within the grafts by 3–5 days after operation.

**Administration of retinoic acid.** Retinoic acid (RA) was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 50 μg per μl, and used fresh. As diagrammed in Fig. 1, each group of animals was divided into (1) an experimental subgroup which was injected intraperitoneally, via microliter syringe, with 150 μg of RA per gram of body weight at 4 days post-amputation, and (2) a control subgroup which was not injected. DMSO controls were not done because we have shown previously that injection of DMSO alone has no effect on axolotl limb regeneration (Thoms and Stocum 1984; Kim and Stocum 1986).

**Histology.** Control regenerates of normal limbs were fixed in Bouin’s solution at 2 day intervals from 2 to 20 days post-amputation, and RA-treated regenerates of normal limbs were fixed at 2–3 day intervals from 1 to 23 days.