least 60 days, an interval sufficient for well-formed four-digit regenerates to develop in the normal sequence for this species. At the time of sacrifice, both the experimental and contralateral control limbs were dissected free at the shoulder and fixed in Bouin's solution. They were evaluated grossly for regenerative success then decalcified and prepared for paraffin embedding, serially sectioned at 8 μm and stained by Mallory's trichrome method. Control and experimental limbs were then compared, evaluating: a) skin morphology as an indicator of graft survival and contribution to the regenerate and b) skeletal composition, especially number of digits and internal cartilaginous elements as an indicator of morphogenetic success.

A total of 41 animals were evaluated providing 22 limbs from the inverted tail skin group and 19 from the normally oriented tail skin group.

RESULTS AND DISCUSSION

Simple amputation of the control left limbs usually resulted in the regeneration of normal 4-digit limbs. Histologically, these limbs were covered by typically thin, sparsely glandular skin and contained the cartilaginous precursors of a normal skeletal complement. Limbs which had received grafts of tail skin prior to amputation yielded a range of responses from no regeneration to a superficially normal 4-digit regenerate. Skin graft orientation, whether normal or inverted, had no apparent influence on the results. Stumps which did not regenerate had histologically obvious, thick glandular skin about their entire circumference. Many examples, where tail-type skin differentiated on the regenerate, had reduction deformations (e.g. fewer digits than normal; distally fused radius and ulna). Those with the most complete regenerates often had an incomplete circumference of tail skin around the stump region. This may have been due to graft resorption or localized gland regression, either of which produces a "wound epithelium" which is more supportive of regenerative events than whole skin. None of the experimental combinations produced chimeric regenerates wherein tail-like structures would appear either grossly or histologically in the limb regenerate. This latter observation is contrary to the earlier studies of Glade (J. Morph. 101:477-522, 1957; Growth 42:253-262, 1978) who, using aquatic species which have tail fins, described fin and tail-like structures on limb regenerates from stumps bearing tail skin grafts. While this may indicate fundamental differences between species, it is a likely reflection of graft contamination with non-skin tissues which express morphogenetic activity or, at least, the capacity to self-differentiate in a heterotopic locus.

The results of the present study indicate that, at best, tail skin may be mildly supportive of limb regeneration causing varying degrees of morphogenetic disruption within the host field. However, in many cases, regeneration was completely inhibited. In those limbs which did regenerate and bore tail skin around the stump at the time of sacrifice, there is no evidence for autonomous morphogenetic contributions of tail structure to the regenerate. It therefore appears that, unlike limb skin, tail skin plays a passive to supportive role in normal epimorphic events. When heterotopically grafted, it interferes with normal morphogenetic signalling in the host field without making an autonomous contribution of morphogenetic information.

A STUDY OF THE KNOWN LIMB TERATOGEN, ACETAZOLAMIDE, AND ITS EFFECT ON LIMB REGENERATION

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Vertebrate limb development and urodele limb regeneration appear to have much in common and because of their structural and sequential similarities, the latter is frequently said to recapitulate the former (Faber Adv. in Morph.,

9:127-147, 1971; Stocum, Differentiation, 3:167-182, 1975). It is also inferred from such observations that obligatory morphogenetic tissue interactions in the two systems may be analogous; several recent models on pattern regulation make this assumption. Nature being generally conservative once a workable system is established, one would expect the foregoing assumptions to be borne out. The hypothesis that the two systems employ the same regulatory mechanisms may be tested by studying the manner in which a well-defined chemical teratogen known to disrupt one of the systems affects the other system.

The present investigation examines the effect of acetazolamide on various stages of salamander limb regeneration. This specific and potent carbonic anhydrase-inhibitor has been repeatedly shown to induce post-axial forelimb deletions in embryonic rodents expression of the defect being dose- and stage-dependent. Although acetazolamide-induced limb anomalies were first observed over 15 years ago (Layton and Halsey, Sci. 149:306-308, 1965), the primary site of the drug effect in this system remains unknown (Holmes and Trelstad, Teratology 20:289-296, 1979). The main purpose of this study is, therefore, to determine whether or not epimorphic regeneration of the urodele limb, like limb development is susceptible to morphogenetic disruption by acetazolamide. A positive result would, in addition to supporting the above assumption, provide an experimental model with greater access and ease of experimental manipulation than mammalian embryos for elucidating the mechanism of drug action at this locus of morphogenetic interactions. A negative result would indicate likely autonomous mechanisms for pattern regulation in developing and regenerating limbs with serious implications for previous and future comparisons of these developmental systems.

MATERIALS AND METHODS

All of the following procedures were performed on Eastern red-backed salamanders, Plethodon cinereus, collected in the vicinity of the Laboratory in Salsbury Cove. Specimens were paired by size and maintained in 4-inch diameter stacking culture dishes containing moistened paper toweling and at ambient laboratory temperature. They were fed regularly with live fruit flies (Drosophila melanogaster) and cleaned as needed. The ninety-four animals in this experiment ranged from approximately 0.7 to 1.5 grams total body weight.

The preliminary procedure was to initiate limb regeneration by amputating both forelimbs through mid-humerus. Groups of 8 or 10 animals were individually anesthetized in 1% MS 222 (ethyl m-aminobenzoate methanesulfonate, Eastman). Following limb amputation and soft tissue retraction, the protruding segment of the humerus was trimmed and the animals returned to their bowls for recovery. At selected post-amputational times (6-14 days), the groups were begun on a regimen of intraperitoneal injections of acetazolamide (Diamox, Lederle Laboratories Division, Pearl River, New York) twice daily (10:00 AM and 4:00 PM) for 3 consecutive days. Toxicity of the drug for this species was tested over a range of 30-1000mg/kg. The lower doses (30 and 100mg/kg) were approximately at the LD_{50} but survivors from the higher doses (39% at 300mg/kg; none at 1000mg/kg) were also evaluated for regenerative success. The drug was delivered I.P. at concentrations of 1% (pH 8.3) and 0.3% (buffered to pH 7.8) through a 30 gauge needle on a Hamilton microliter syringe. It was necessary to anesthetize the animals lightly at the time of injection in order to control them appropriately and avoid tail autotomy (Dinsmore, Bull. MDIBL 16:22, 1976).

Observations on the responsiveness of animals during the post-injection period were recorded and the initiation and progress of the regenerative response followed for 45 to 50 days post-amputation. At that time, animals were anesthetized and both forelimbs removed at the shoulder, fixed in Bouin's solution and subsequently scored for regenerative success.

RESULTS AND DISCUSSION

Plethodon cinereus is a terrestrial, lungless salamander and appears to be more sensitive to the effect of carbonic anhydrase inhibition than the mammals in earlier studies. Doses which produced no reported toxicity to pregnant female rodents were lethal to the salamander. Even the lower doses (30 and 100mg/kg) produced a high mortality rate at 10 days after initial drug injections (47% and 44% respectively). Although we have not yet explored this observed high toxicity, several explanations are immediately evident. In healthy mammals with unimpaired lung function, the chief compensation for inhibition of red blood cell carbonic anhydrase is diffusion of gaseous CO_2 across lung epithelium (Swenson and Maren, Resp. Physiol. 35:129-159, 1978). It is therefore, not surprising that lungless salamanders would show elevated sensitivity to a carbonic anhydrase inhibitor. Furthermore, the main route of acetazolamide excretion in all species studied thus far is the kidneys and in lower vertebrates (e.g., dogfish sharks), this is a relatively slow process (Maren, Comp. Biochem. Physiol. 5:201-215, 1962). Therefore, it is likely that high systemic levels of the drug are reached with the present protocol though we do not yet know the fate of acetazolamide in this species.

Twenty-nine of the 68 animals whose limbs were amputated survived the 50 day post-amputation period. These represented specimens receiving drug injections on days 6-8, 8-10, 11-13 and 14-16 of regeneration. These periods were selected as times when critical morphogenetic interactions, which would be comparable to those disrupted by acetazolamide in 9-10 day rodent embryos, occur (Holmes and Trelstad, op. cit). Furthermore, they cover the period which appears thalidomide-sensitive during newt limb regeneration (Bazzoli et al., J. Embryol. exp. Morph. 41:125-135). Each of the experimental animals in these groups possessed bilateral regenerates demonstrating that acetazolamide does not block the initiation of the epimorphic response nor subsequent outgrowth of the regenerate.

Morphogenesis of the regenerate progressed normally in a vast majority of the pooled regenerates. Seventy-four percent had superficially normal, 4-digit hands. An additional 23% were at late blastema to late paddle stages of regeneration which, though delayed, is within the normal range of variability for this 45-50 day regenerative period. Subsequent histological analysis of precartilage patterns in these regenerations will likely resolve some of them into specific digital pattern commitment. Two of the 58 limbs analyzed have surface indications of only three digits each. While this represents a 3% rate of gross malformation, Dearlove and Dresden (J. Exp. Zool. 196:251-262) found that over 6% of ordinary newt limbs will produce abnormal regenerates. A comparable study has not been done for this species, but a low percentage of anomalous regenerates is probable. Since one was from the 300mg/kg group and the other from a 30mg/kg group, it is unlikely that any reduction abnormality is related to a specific drug effect.

We conclude that while acetazolamide is more toxic to adult Plethodon cinereus than to adult rodents, it does not disrupt morphogenesis of regenerating salamander limbs. Since comparable doses of this drug create specific limb defects in mammalian embryos, the regenerating urodele limb may not be a good analog for limb development. It is suggested that at least some components active in morphogenetic information transfer in these two systems are unique. Further exploration of acetazolamide-induced limb anomalies may clarify these inductive mechanisms.

FURTHER OBSERVATIONS ON TWINNING IN THE SPINY DOGFISH

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In 1976, we reported the first observation of twinning in the spiny dogfish (A.D. Woodhead, Bull. MDIBL 16:106), one instance in 270 adult female fishes samples. Although it has generally been concluded that developmental abnormalities are rare in elasmobranchs, as compared with teleosts, we found a second case of twinning this