Participation of grafted nerves in amphibian limb regeneration

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SUMMARY

Irradiated axolotl arms bearing grafts of brachial nerves can regenerate, albeit imperfectly. The origin of the regenerated tissues has been traced by means of the difference between dark and white genotypes. Although the origin of the melanophores cannot be decided in this way, their abundance identifies the genotype of the tissue they occupy. Sections of nine regenerates provide consistent evidence for the following conclusions. (1) The epidermis of the regenerate is probably an extension of the irradiated host epidermis. (2) All other tissues of the regenerate which differentiate from a mesenchymal blastema originate from the non-irradiated graft. Regeneration thus involves a true dedifferentiation and redifferentiation (i.e. a loss of determination and equivalent gain in potency for histological transformation). Irradiated cells apparently also dedifferentiate but cannot undergo the normal sequence of proliferation and redifferentiation, and so are not found in the mesodermal component of the regenerate.

INTRODUCTION

It has been known for many years that limbs of urodeles and anuran tadpoles regenerate almost perfectly after amputation, but can be prevented from doing so by sublethal doses of ionizing radiation. Most relevant observations concern the effects of X-rays, applied either before or immediately after amputation (reviewed by Thornton, 1968). The X-rays act directly on the cells exposed to them and the regeneration blastema normally arises from tissues very close to the site of amputation, so that localized irradiation also inhibits regeneration. If the amputation site has been previously irradiated, normal wound-healing still occurs by an epithelial migration and some of the internal tissues liberate dedifferentiated cells, but these irradiated cells do not accumulate as a blastema. The irradiated limb-stump may then remain as a permanent feature of adult urodeles, but gradually regresses in larvae by the continued dedifferentiation and erosion of the skeletal apex. If an irradiated limb carries a graft of non-irradiated tissue just proximal to the amputation site, it usually regenerates. The nature of the regenerated structure depends to some extent upon the origin of the graft, but in the best cases (cited by Conn, Wessels & Wallace, 1971), a structurally and functionally normal limb can be obtained. Nobody has yet demonstrated precisely what is responsible for this recovery of regenerative

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ability. Either the grafted cells provide all the tissues of the regenerate, or they somehow elicit a recovery of neighbouring irradiated host cells which then form the principal source of the regenerate. Rose & Rose (1965) have argued in favour of the latter explanation which was apparently adopted earlier by Vorontsova & Liosner (1960), although the bulk of opinion and perhaps of evidence supports the former explanation (Umanski, 1937; Thornton, 1968; Wallace, 1972).

The only experimental means of distinguishing between these explanations is to have the host and graft differently marked or labelled, so that cells of the regenerate can be assigned to one source or the other. Radioactivity is not a suitable marker in this situation, as the label is diluted by half at each cell-division. In practice, such labelled cells have not been traced for more than four successive divisions in a blastema (Oberpriller, 1967). The use of polyploid graft tissue provides a permanent identification of some cells in the regenerate (Bliakher & Kraskina, 1949; Steen, 1968), but the origin of the majority of cells is not easily distinguished on this basis. The natural colour difference between dark and white axolotls is sufficiently distinct to mark grafts and has the practical advantages of operational simplicity and permanence. The chief drawback of this genetic marker is that its use can only be justified by an elaborate theoretical analysis.

A single gene controls this colour difference, dark (D) being dominant to white (d), by regulating the number of melanophores which occupy the dermis and surround the blood vessels. White axolotls (dd) possess relatively few melanophores, mainly distributed about the dorsal midline, while one or two melanophores can occasionally be found in the limbs. The distribution of other pigment cells is equally restricted in white axolotls and can be ignored for the present purpose. The gene only indirectly affects melanophores: the migration, proliferation and expansion of all melanophore genotypes are seriously impaired by contact with dd tissue (Dalton, 1950; Brick & Dalton, 1963). Direct observation, moreover, cannot preclude the possibility that cryptic melanoblasts may migrate through any host tissue, be present in any graft, and so enter a regenerate from either source. Indeed, there is already evidence that melanoblasts are able to multiply and migrate from one tissue to another (Dalton & Krassner, 1956; Duncan, 1961). Even though the origin of melanophores in a regenerate cannot be established, there is the clear expectation that the abundance of melanophores will be related to the genotype of the tissue they eventually occupy. DD or Dd tissue will be densely populated by an abundance of melanophores; dd tissue will be marked by the virtual absence of melanophores. The significance of this distinction is considered below in terms of the regenerates formed on irradiated arms which contain non-irradiated grafts, produced by transplanting unpigmented brachial nerves reciprocally between dark and white axolotls.

I. Dark host, white graft. (A) An abundance of melanophores in the regenerate
Graft-specific regeneration

could only be supported by the dominant gene, indicating the presence of irradiated host cells. (B) The absence of melanophores from the regenerate could be achieved either if it was derived entirely from dd graft tissue, or if melanoblasts could not enter the regenerate (which then might be of host or graft origin).

11. White host, dark graft. (C) An abundance of melanophores in the regenerate could only be supported by the dominant gene, indicating the presence of graft cells. (D) The absence of melanophores from the regenerate could be achieved either if it was derived entirely from irradiated host tissue or if melanoblasts could not enter the regenerate (which might then be of host or graft origin).

Assuming a consistent coloration of the regenerate is obtained repeatedly from each of these reciprocal graft situations, the same explanation must hold for each. The paired results should thus fall into one of the following predicted categories, at least three of which reveal the genotype of the regenerate.

**Prediction 1.** If all regenerates are pigmented (A and C), then they must be a mixture of both host and graft cells; such pigmentation might be diluted or patchy (cf. Schaxel, 1922).

**Prediction 2.** If all regenerates resemble the host coloration (A and D), then they are derived entirely from host cells, which thus must have recovered from irradiation.

**Prediction 3.** If all regenerates are coloured like the graft donors (B and C), they must be derived entirely from graft cells. As the latter form only a minor proportion of the cells near the site of amputation, this implies that irradiated host cells cannot participate in regeneration.

**Prediction 4.** If all regenerates are colourless (B and D), then their origin remains ambiguous. This prediction implies that melanoblasts could not reach, survive or differentiate in the regenerate. Anton (1965) has already shown that other pigment cells can migrate from a host to a grafted regenerate, and our results suggest this may be true for melanophores.

In principal, these predictions should hold for any tissue graft which can promote the regeneration of an irradiated limb. There are, however, two important qualifications to the analysis. First, the definitive pigmentation might be delayed until after the morphological completion of the regenerate, especially where melanoblasts have to migrate from the trunk of a white host in order to reach the regenerate. Predictions 2 and 4 might therefore require an independent control of the rate of pigmentation under similar conditions. Secondly, different tissues of the regenerate might produce results in accordance with different predictions. By repeatedly amputating regenerates which had been orthotopically transplanted between dark and white axolotls, David (1932) showed that host epidermis and dermis could migrate distally over the internal tissues. Similar movements of limb skin have been reported since (see Wallace, Wessels & Conn, 1971). It follows that conformity to these predictions must be assessed independently for each tissue, preferably in sectioned material.
An initial test of these predictions yielded the required consistency of results among five $Dd$ hosts with $dd$ nerve implants and four $dd$ hosts with $Dd$ nerve implants (Wallace, 1972). Records of the progress of pigmentation in the experimental and control regenerates suggest that the pigmentation of the skin conforms to prediction 1, while the internal elements of the regenerates conform to prediction 3 and thus have originated from the Schwann cells and fibroblasts of the grafts. We have now examined the distribution of melanophores in sections of these regenerates. We believe our observations vindicate the preceding theoretical analysis and justify the conclusions summarized at the beginning of this article.

**MATERIALS AND METHODS**

The operations were performed on 70–80 mm axolotls, *Ambystoma mexicanum*, $Dd$ and $dd$ sibs of a single mating between a white female descended from Dr Humphrey's stock at Bloomington, Indiana, and a heterozygous dark male of unknown ancestry obtained locally. Assuming a history of inbreeding in both these parental lines, the $F_1$ should be identical, even if heterozygous, at most allogenic loci (histocompatibility genes). This and their comparative youth should avert any immunological rejection of grafts, such as described by de Both (1970). All grafts were retained.

The entire left arm and shoulder of each potential host specimen were irradiated with 2 krad (20 J/kg) of X-rays. A length of cleaned brachial nerve from a non-irradiated donor of different genotype was then inserted into the left forearm and allowed to heal in for 3 days. The arm was then amputated just below the graft, and the shielded right forearm was also amputated to provide a control of the normal rate of regeneration. Duplicate examples of the grafted nerves were sectioned, and found to contain only axons, Schwann cells and connective tissue cells. Details of this procedure have been published separately (Wallace, 1972). That report also includes evidence that the irradiation employed uniformly inhibits regeneration, and that the regeneration supported by these grafts is retarded in comparison to the control right arms.

When the experimental regenerates were judged to have attained a stable pigmentation, well after the completion of morphological regeneration, they were fixed in Bouin, cut into serial sections and stained with Harris haematoxylin and eosin.

**RESULTS**

Our observations essentially compare the regeneration of experimental left arms to control right arms. The latter regenerated without delay and so were larger and more mature at the time of fixation. Sections of these control arms revealed a range of minor anatomical defects, such as the occasional fusion of cartilages. To simplify the description, only major defects of the experimental arms are noted, together with the distribution of melanophores in the sections.
Graft-specific regeneration

This is preceded in each case by a brief account of the course of regeneration and pigmentation in the experimental arms.

Two general features of the regenerates are recorded here to avoid repetition in the case-histories. First, the pigmentation of dark control limbs is most pronounced on the upper surface of the hand and arm. Sections show a corresponding variation in the abundance of dermal melanophores. Virtually all melanophores were found to be associated with either the dermis or blood vessels (Figs. 1, 2); they occurred so rarely in the epidermis of even dark control arms that we could not distinguish the genotype of the epidermis. Second, the specimens were fixed when about 10 months old and their regenerates had been fully formed with digits for over 3 months. Consequently, all regenerates differed from the arms of young larvae in several respects: the epidermis was several cells thick; the dermis was conspicuous, with glandular regions as thick as the epidermis; central regions of the cartilages had been eroded by blood vessels (Fig. 1C), perhaps as a prelude to ossification (cf. Trampusch & Harrebomée, 1965; Bloom & Fawcett, 1968, fig. 10–25).

White hosts with dark grafts

Four specimens survived from five operations of this kind. All the control limbs regenerated normal hands with four digits within 4–5 weeks of amputation. Both arms of cases 1–3 were fixed after 24 weeks.

Case 1. The left arm showed a cone-shaped blastema 4 weeks after amputation. The cone had elongated by 7 weeks and then contained a row of six melanophores, which had not been observed anywhere on the left arm up to that time. The left regenerate formed four digits by 10 weeks and continued to grow, but remained smaller than the control right hand. All the left regenerate was generally pigmented after 10 weeks, showing numerous superficial discrete melanophores over a yellow-grey background produced by deeper xanthophores and melanophores and thus attaining the typical appearance of a dark limb. The striking pigmentation of the living specimen has been recorded previously (Wallace, 1972; fig. 4A). Sections of the experimental forearm showed that either the radius or ulna was abnormally short, while the musculature was restricted to a few small bundles of fibres surrounded by general connective tissue. The melanophores, however, were distributed around the dermis and the majority of blood-vessels at approximately the same density found in dark control arms.

Case 2. This duplicated the previous case both in the timing of regeneration and in the complete pigmentation of the left regenerate (Fig. 1A). Sections of the left forearm revealed distal fusion of the radius and ulna, and a deficiency of muscles similar to that described for the previous case. Melanophores surrounded most of the blood-vessels and occupied the dermis at a high density in part of the sections and at lower densities elsewhere (Fig. 2A).

Case 3. The left arm regenerated rather more slowly than the two previous
cases, showing four digits after 12 weeks. The left wrist was twisted to give the hand a supine posture. The entire left regenerate became well pigmented but not so dark as the previous cases. The right regenerate was also malformed, bearing an excrescence at the wrist which resembled an incipient duplication without any digits. Sections of the left forearm showed that the radius and ulna remained well separated down to a malformation at the twisted wrist. Both cartilages were surrounded by thick perichondrial sheaths and partly infiltrated by blood vessels. Massive blocks of muscle occupied most of the area in each section, giving this regenerate a fairly normal appearance. Melanophores were present as patches in the dermis, almost exclusively on one side of the arm, and also generally distributed about the blood vessels. This experimental arm may be compared to the control right arm in Fig. 1 and C, D.

Case 4. The left arm failed to regenerate, but regressed to slightly above the original position of the graft during 10 weeks. A new accumulation of about 17 melanophores then appeared on the dorsal surface of the elbow and the entire forearm stump gradually became deeply pigmented during the following 8 weeks. A savage reamputation, removing all scar tissue and most of the pigmented elbow, provoked an abortive form of regeneration - a cone blastema which became bifurcated and pigmented at 10 weeks after reamputation, but had not changed noticeably at 20 weeks when it was removed and fixed. Sections of this material disclosed a single element of cartilage embedded in poorly organized muscle. Melanophores occupied all parts of the dermis and the deeper connective tissue, being present even in regions where no blood vessels were apparent. While failing to meet our usual criteria of regeneration, this specimen conformed to the previous cases in showing growth, differentiation and deep pigmentation.

Dark hosts with white grafts

All five specimens survived and regenerated both arms. The control right arms formed virtually normal hands with four digits within 4–5 weeks of amputation and became completely pigmented during further growth. Both experimental and control regenerates were fixed 24 weeks after amputation.

Case 5. The left arm reached a normal four-digit stage in 12 weeks, by which time it showed nearly 100 superficial contracted melanophores but had no deeper pigmentation. The regenerate remained rather smaller and much paler.

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Fig. 1. (A, B) Live specimens 24 weeks after amputation. See text descriptions of case 2 for (A) and case 8 for (B). The colour transition in the left arms lies proximal to the base of the regenerate. (C, D) Sections of left and right forearms of case 3. Melanophores clearly surround the blood vessels of (C) but are scarcely detectable in the dermis, and are entirely absent from (D). (E, F) Sections of left and right forearms of case 7. Melanophores are prominent in the dermis and surround the blood vessels of (F), but none occur in (E). Note the similar formation of muscle and cartilage in each section.
than the control right arm up to the time of fixation. Sections of the experimental arm showed a normal disposition of the radius and ulna and other cartilages in the wrist and hand, but only a few small bundles of muscle fibres separated by large areas of connective tissue. Melanophores occurred sparsely in the dermis around three-quarters of the circumference of the forearm, but were entirely absent from the central blood vessels.

Case 6. The left arm formed a rather slender regenerate with the normal four digits. The rate of regeneration and the number of exclusively superficial melanophores corresponded closely to the previous case. The radius and ulna were found to be fused in distal sections of the arm, which contained very few strands of muscle embedded in general connective tissue. The dermis contained some melanophores, which were also occasionally found in the epidermis and on one blood-vessel.

Case 7. The left arm formed a partially duplicated regenerate bearing six digits. The entire regenerate contained only 23 superficial melanophores at 12 weeks, and remained virtually white up to 24 weeks, after amputation, as the few melanophores were extremely contracted (see Wallace, 1972; fig. 4B). Sections of the experimental forearm revealed a fairly normal anatomy: the separate radius and ulna were embedded in blocks of striated muscle. Although occasional melanophores were detected in the dermis, most sections showed no pigment and thus could not be distinguished from sections of normal white arms. Sections from both arms of this specimen are shown in Fig. 1E, F.

Case 8. The left arm regenerated a perfectly formed hand, as large as the control at 12 weeks, but the wrist later became permanently flexed so that the back of the hand was used for walking. The entire left regenerate was estimated to possess 200 superficial melanophores at 12 weeks. These gave the regenerate a freckled appearance owing to the absence of deeper pigmentation which is characteristic of the control limbs (Fig. 1B). Sections revealed the virtual absence of muscle from this experimental regenerate: isolated fibres which resembled muscle fibres but lacked cross-striations lay close to both the radius and ulna. The melanophores provided a long patch of dermal pigment and a small patch around one blood vessel.

Case 9. The left arm regenerated at the same rate as the previous cases but only produced three digits. The entire left regenerate contained about 100 superficial contracted melanophores at 12 weeks. This number increased slightly during later growth but no deeper pigmentation developed. Sections of the left forearm showed that the radius and ulna had completely fused into a single cartilage, partly eroded by blood vessels and adjacent to a few small bundles of muscle fibres. As in the previous case, one side of the arm contained more dermal melanophores than were encountered in the white control arms but fewer than occur in dark controls. No melanophores were found on the internal blood vessels.
DISCUSSION

The theoretical analysis set out in the Introduction required consistent results from reciprocal grafts. Our results from nerve grafts are sufficiently consistent for that purpose. Although the dose of irradiation employed here completely prevents regeneration (Wallace, 1972), the present cases all regenerated to some extent and most of them achieved the normal four digits. In the context of previous similar operations (e.g. Umanski, 1937), there is no doubt that the non-irradiated grafts were directly or indirectly responsible for restoring a regenerative competence to these irradiated arms. Despite the wide spectrum of anatomical defects in these regenerates and their general inferiority to the control regenerates, the best cases demonstrate beyond question that all normal tissues of the arm can be regenerated in fairly normal amounts and arrangement. The colour of the experimental regenerates is also reasonably consistent within each graft series, and quite distinct from the colour of the control regenerates. The restricted distribution of melanophores, as observed especially in sections, limits the number of regenerated tissues whose genotypes can be identified directly. The epidermis of both experimental and control regenerates contained so few melanophores that it appeared identical in all cases. Recalling that the host epidermis rapidly covers the wounds caused by grafting and amputation, and appears to remain intact thereafter, we have no reason to question the generally accepted conclusion that all regenerates are covered with host epidermis. The dermis of the experimental regenerates of both series held a rather variable population of melanophores, usually less than in dark control arms but always clearly more than in white controls. These melanophores lay superficially under the transparent epidermis, permitting their numbers to be estimated in living specimens. Dark hosts with white grafts carried 200 or less dermal melanophores in the entire experimental regenerate. The corresponding regenerates formed by white hosts with dark grafts always showed far greater numbers and densities of dermal melanophores (Fig. 2A). We conclude that the dermis of such regenerates shows essentially the characteristics of the graft genotype; it might include a minor proportion of host cells or, more probably, the abundance of melanophores there is slightly influenced by the adjacent host epidermis. The internal melanophores were usually found in contact with blood vessels. We must therefore associate these melanophores principally with the genotype of the blood vessels, although the adjacent tissues could well influence their density. The distribution was not uniform; patches of melanophores occurred occasionally on the blood vessels of white control arms, while dark controls frequently contained lengths of blood vessels with few or even no melanophores. As far as can be judged from the general intensity of pigmentation in living specimens and from the density of melanophores in sections of the experimental regenerates, the abundance of internal melanophores corresponded precisely to the genotype of the grafts (Figs. 2C–F). We conclude that the blood vessels,
Fig. 2. (A) Dermal melanophores in the left forearm of case 2. (B) Dermal melanophores in the right forearm of case 7. (C–F) Blood vessels from the identically lettered photographs of Fig. 1, enlarged to show that only the blood vessels of C and F are surrounded by melanophores.
at least, are exclusively derived from the grafts. It is also arguable that the general connective tissue must also be derived from the grafts, for otherwise it would reveal the host genotype by modifying the degree of pigmentation on the blood vessels. We regard that as only a supporting argument. All the internal tissues of the regenerate are known to differentiate from the mesenchymal cells of a compact blastema. It would indeed be a strange coincidence if these mesenchymal cells should segregate according to their genotypes, instead of according to their position in the blastema. Since the blood vessels can be identified as originating from the grafts, we are confident that the connective tissue, muscle and cartilage of these regenerates must also be composed entirely of non-irradiated graft cells.

The nerves used for these grafts contained neurilemmal Schwann cells and fibroblasts of the supporting connective tissue layers, but were completely devoid of such easily recognized tissues as cartilage, muscle or blood vessels, besides lacking erythrocytes and melanophores. Our results prove conclusively, therefore, that the dermis and the blood vessels can originate from fibroblasts or Schwann cells. We believe our results also imply that these same cells can transform into cartilage and, with some difficulty perhaps, into muscle. In general terms, dedifferentiation must involve an effacement of histological determination, permitting blastemal cells to redifferentiate into tissues unlike those from which they originated.

The grafts constituted only a minor proportion of the tissue in the region adjacent to the plane of amputation yet, to the extent that can be tested, the graft provided all the mesodermal tissues of the regenerates. We therefore conclude that the irradiated host cells cannot participate in regeneration, apart from providing the epidermis. The latter certainly participates in regeneration but in a manner quite distinct from the mesenchymal blastema (cf. Wallace, 1972). The original question of cellular potency in regeneration is considerably clarified by these results: adequately irradiated cells are nullipotent; non-irradiated cells certainly gain some measure of potency, whose expression is perhaps only limited by the requirements of the regenerate. We anticipate that these conclusions may be challenged, if only because of their wider implications. We defer examining these implications until we have tested the general validity of the analysis, which we are now attempting by means of cartilage grafts.

REFERENCES


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