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Permalink
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Journal
Developmental Biology, 106(2)

ISSN
0012-1606

Authors
Rollman-Dinsmore, C
Bryant, SV

Publication Date
1984

DOI
10.1016/0012-1606(84)90225-2

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The Distribution of Marked Dermal Cells from Small Localized Implants in Limb Regenerates

CHRISTINE ROLLMAN-DINSMORE AND SUSAN V. BRYANT

Developmental Biology Center and the Department of Developmental and Cell Biology, University of California, Irvine, California 92717

Received November 30, 1983; accepted in revised form June 25, 1984

Numerous experiments have demonstrated that skin has a profound influence on the pattern of limb regeneration in urodèles. In this investigation, the fate during regeneration of marked cells derived from narrow strips of skin inserted into different positions around the limb circumference has been followed. Skin strips were taken from triploid axolotls and transplanted into diploid sibling animals. The distribution of trinucleolate cells was determined at the site of amputation and in the regenerated limb. The results indicate that at the time of amputation marked cells appear to be localized to the graft, whereas in the regenerate marked cells may be found at all proximal-distal levels and at any position around the circumference of the limb. These results are discussed in terms of a possible mechanism for distal outgrowth.

INTRODUCTION

Numerous experiments have shown that the skin of the amphibian limb stump can influence the pattern of the regenerate. When skin is grafted into a new position or in a new orientation, the resulting regenerates form a variety of extra structures after amputation through the grafted region (Carlson, 1974, 1975; Lheureux, 1972, 1975, 1977; Rollman-Dinsmore and Bryant, 1982; Settles, 1970; Slack, 1980, 1983; Tank, 1981). Even a small strip of skin added to the limb circumference may produce these results (Rollman-Dinsmore and Bryant, 1982; Tank, 1981). The formation of these abnormal regenerates is dependent on grafting so as to appose cells of disparate circumferential positional values. Control operations which do not appose cells of disparate positional values yield normal regenerates after amputation.

Although various experiments have shown the effect of skin on patterning, only Slack (1980, 1983) has reported on the fate of skin cells from surgically constructed limbs after regeneration. He reported that cells from triploid skin grafts remain localized in structures adjacent to the graft. In the experiments reported here, we have analyzed the distribution of trinucleolate cells in the mature regenerates formed from limb stumps which contained triploid skin grafts in various positions around the circumference of a diploid host limb. The data obtained indicate that contrary to Slack's results, marked cells in the regenerates were not localized to the region adjacent to the graft, but were found distributed around the circumference of the regenerate.

MATERIALS AND METHODS

Experiments were performed on sibling axolotls, *Ambystoma mexicanum*, which ranged in length from 70 to 90 mm, snout to tail tip. All animals were anesthetized in MS-222 (Aldrich) diluted 1:2000 for surgery. Both diploid hosts and triploid donors were obtained from natural spawnings at the University of California, Irvine axolotl colony. Triploid animals were produced by hydrostatic pressure (600 psi for 8 min) using the protocol of Gillespie and Armstrong (1979). Prior to use, pressurized animals were scored for ploidy in bismuth-stained whole mounts of hand tissues of young larvae (Muneoka et al., 1984). After surgery animals were maintained at 20 ± 1°C in individual plastic boxes filled with 25% modified Holfreter's solution. For 1 week after amputation animals were observed daily for the presence of the graft. Thereafter they were observed on a weekly basis.

Strips of skin (dermis and epidermis) 1 to 2 mm wide, were removed from the upper arms of triploid animals. Individual strips, which were approximately one-eighth of the circumference of the limb were then inserted into a prepared longitudinal slit in the upper arm of a diploid host. The edges of the cut host skin were pulled close to the grafted skin so as to make a continuous circle of skin around the internal tissues of the limb. The natural adhesivity of the skin to the underlying tissues held the graft in place. One week after grafting, limbs were amputated through the distal edge of the graft, and then allowed to regenerate to maturity, approximately 1 month later. The limbs were then fixed in Carnoy's, decalcified in Versene (pH 6.5), and embedded in paraffin. Serial transverse sections, 10 μm thick, were stained with the nucleolar-specific bismuth stain (Locke and Huie, 1977) as modified for amphibians (Muneoka et al., 1984).

The sites of origin and transplantation of control skin strip grafts were as follows: anterior into anterior (2), posterior into posterior (2), dorsal into dorsal (2), and ventral into ventral (2); for experimental grafts:
posterior into anterior (2), anterior into posterior (2),
dorsal into ventral (2), and ventral into dorsal (2). The
anatomical designations used were those described by
Tank et al. (1976).

A further group of eight animals was prepared in
the same way as described above, however, their limbs
were amputated close to the proximal edge of the
graft. The amputated portion of the limb was fixed
and prepared for sectioning and staining as described
above. These samples were used to determine the
location of the transplanted cells at the time of am-
putation.

For analysis, the cross sections of each limb were
divided into four equal quadrants designated as 45,
135, 225, and 315 (see Fig. 1). These four quadrants
were chosen to approximate an equal distribution of
dermis in each, based on the data of Tank and Holder
(1979). The graft always overlapped the boundary
between the 45 and 315 quadrants. Regardless of the
type of graft, i.e., anterior into anterior, posterior into
anterior, etc., the quadrants were designated such that
the 45 quadrant was set as the first quadrant clockwise
to the graft followed by 135, etc. Only the cells of the
dermis were examined for the presence of trinucleolate
cells. The number of cells with three nucleoli in the
dermis of the four quadrants was determined for both
zeugopod and autopod. Counts were made on equal
numbers of scattered samples of sections in the prox-
imal, middle, and distal portions of the zeugopod and
the proximal and middle portions of the autopod.
Counting was continued until at least a total of 50
trinucleolate cells were scored in each segment of the
limb. The percentage of trinucleolate cells in a quadrant
was determined by dividing the total number of trinu-
ucleolate cells of a quadrant of a limb segment by the
total number of trinucleolate cells for that particular
limb segment and multiplying by 100.

The limbs of a group of diploid animals were prepared
as above to determine the background level of apparent
triploid cells in diploid animals. The average number
of trinucleolate cells in regenerates derived from diploid
limbs was only 0.18 cells per section, whereas the
number for regenerates derived from diploid limbs
containing grafts was 3.21 cells per section.

RESULTS

Axolotls, like newts (Rollman-Dinsmore and Bryant,
1982), formed supernumerary skeletal elements when
skin was transplanted so as to appose cells of maximum
circumferential disparity (Fig. 2). Sixty-three percent
of the experimental limbs formed supernumerary digits
(Table 1). All control limbs formed normal regenerates
without supernumerary elements.

In both experimental and control limbs, the marked
cells which originated at the border of the 45 and 315
quadrants gave rise to marked cells which were dis-
tributed in all four quadrants of the circumference in

**Fig. 1.** Transverse sections of a regenerate from a limb which
received a transplant of an anterior strip of triploid skin into an
anterior site on a diploid limb. (a) Section through the base of the
autopodium. Dorsal is at top and anterior is to the right of the
figure. Graft is centered around arrow. Black lines indicate division
into quadrants. The first quadrant clockwise to the graft is the 45
quadrant. The other quadrants follow in order, moving clockwise
around the circumference: 135, 225, and 315. Bismuth stain. ×45.
(b) Dermis of 225 quadrant to show cell with three nucleoli (arrow).
Same limb as illustrated in (a). Bismuth stain. ×475. (c) Dermis of
315 quadrant to show cell with three nucleoli (arrow). Same limb as
illustrated in (a). Bismuth stain. ×475. (d) Dermis of 135 quadrant
to show cell with three nucleoli (arrow). Same limb as illustrated in
(a). Bismuth stain. ×475. (e) Dermis of 45 quadrant to show cell
with three nucleoli (arrow). Same limb as illustrated in (a). Bismuth
stain. ×475.
DOUBLE-GREE ET AL. Marked Dermal Cell Distribution

both the zeugopod and the autopod (Fig. 3). In all the
regenerated limbs marked cells were distributed more
or less equally among all four quadrants, regardless
of the position of the graft. Hence for the experimental
limbs, data for all graft positions were summed to-
gether, and similarly for controls, data for all graft
positions were summed. For example, in Fig. 3 the
data in the far left corner represents the pooled values
for the zeugopod from eight control limbs (anterior
into anterior (2), dorsal into dorsal (2), etc.). An
analysis of variance among means showed there was
no significant difference in the distribution of triploid
cells among the quadrants of either control or experi-
mental limbs (0.10 > P > 0.05). Furthermore, there was
no significant difference in the distribution of the
triploid cells between experimental and control limbs
(0.10 > P > 0.05). In contrast to the distribution of
marked cells in the regenerates, grafted cells are
significantly localized in the amputated segment of the
limbs (Fig. 4), where the majority of the cells with
three nucleoli are found in quadrants 45 and 315, that
is, in the quadrants containing the graft.

FIG. 2. Whole-mount preparation of a regenerate formed on a left
limb stump. Prior to amputation the host diploid limb received a
transplant of a posterior strip of triploid skin at an anterior site.
The resulting limb bears a total of seven digits. Ventral view,
Victoria blue B stain. ×15.

DISCUSSION

This experiment has shown that a narrow strip of
skin from the upper arm of a triploid axolotl, when
transplanted into the upper arm of a diploid axolotl,
influences the pattern of the regenerate after ampu-
tation through the grafted region in the same manner
as previously reported for the newt (Rollman-Dinsmore
and Bryant, 1982). When the skin graft is transplanted
to a site 180° opposite to its original site and the limb
is subsequently amputated through the graft, the en-
suing regenerate forms supernumerary digits in a
majority of the cases. In contrast, when limbs contain
grafts which are placed in a site homologous to their
site of origin, only normal regenerates form after
amputation through the graft.

Regardless of whether or not supernumerary struc-
tures are formed, the histological analysis of the lo-
ication of cells derived from the triploid grafts has
shown that marked cells are widely distributed in the
final regenerate. Hence cells from one part of the
circumference can give rise to progeny at all positions
around the circumference and at all proximal-distal
levels, whatever the position of origin and the position
of insertion of the graft. We believe these results are
a reflection of the underlying cellular behavior involved
in blastema formation and outgrowth. It has been
proposed previously that distal transformation is
brought about by a process of circumferential inter-
calation at the wound site (Bryant et al., 1981). This
process is thought to occur as follows: Cells of the
dermis dedifferentiate, maintaining their positional
values, and migrate toward the center of the wound
surface. In so doing, cells from one region of the
circumference will contact cells from distantly located
regions. Interactions between cells with disparate cir-
cumferential values are thought to stimulate cell di-
vision, which leads to the intercalation of cells with
intermediate positional values. Hence the final out-
growth is seen as a product of circumferential inter-
calation coupled with the distalization of newly formed
cells.

Two aspects of the results reported here are consis-
tent with this view. First our results strongly suggest
that dermal cells entering the blastema maintain their
positional values. Thus when grafts were made to sites
180° away from their normal position, cells with dis-
TABLE 1

<table>
<thead>
<tr>
<th>Type of graft</th>
<th>Number of cases</th>
<th>Number of normal limbs with supernumerary digits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>8 (100%)</td>
</tr>
<tr>
<td>Experimental</td>
<td>8</td>
<td>3 (37%)</td>
</tr>
</tbody>
</table>

TABLE 1

COMBINED RESULTS OF GRAFTING TRIPLOID SKIN STRIPS FROM DIFFERENT POSITIONS IN THE UPPER ARM CIRCUMFERENCE TO DIFFERENT POSITIONS IN THE UPPER ARM OF A DIPLOID ANIMAL.
parate positional values are brought into contact. Interactions between these cells will lead to additional circumferential intercalation, over and above that which is occurring during normal outgrowth. The extra cells generated by the confrontation between graft and host will participate in the process of distalization and outgrowth, yielding supernumerary structures in the final regenerate. When grafts are placed in homologous positions in the limb circumference no additional intercalation is stimulated and no supernumerary structures are formed. Second, the distribution of marked cells in the final regenerates of both experimental and control limbs, lends support to the view that cell migration leading to cell mixing and followed by subsequent intercalation, plays a crucial role in regeneration. Hence during the early stages of wound healing and blastema formation marked dermal cells from one part of the circumference are thought to migrate toward the center of the wound surface, coming into contact with unmarked cells of disparate positional values. Intercalation between the confronted cells will lead to the formation of marked cells with new positional values. Depending on the details of wound healing, and on which cells contact one another, the newly intercalated cells could have positional values equivalent to any position around the circumference. As outgrowth proceeds, marked cells will also spread through the different proximal-distal levels. Given a mechanism of this type, one would not expect the distribution of marked cells in regenerates with supernumerary structures to differ from that in regenerates without them, since in each case initial migration and intercalation will lead to the assignment of new positional values to the progeny of marked cells.

One additional feature of these results which should be mentioned is that they argue against sorting out between cells with different circumferential positional values. Although the evidence suggests that cells are migratory during the early phase of blastema formation, in the experimental limbs cells of graft origin do not sort to their appropriate position in the circumference. If they did, supernumerary elements would not form. Indeed, if during outgrowth from a normal stump, cells preferentially made contact with their normal neighbors, no intercalation of new positional values would be stimulated and no outgrowth would ensue.

The idea that blastema formation involves cell migration is not new (Bryant and Baca, 1978; Chalkley, 1954; Thornton, 1968), but cell movement has not been observed directly. However, both scanning and transmission electron microscopic observations of blastema cells (Bryant et al., 1971; Geraudie and Singer, 1981; Norman and Schmidt, 1967; Oberpriller and Oberpriller, 1978) have revealed elongated cells with long filopodia, a morphology which is consistent with that of other motile cells (for review, see Trinkaus, 1976). Further, the extracellular matrix present in early regenerates...
Fig. 4. Histogram of percentage of all trinucleolate cells in the limb circumference which are found in each quadrant in the region of amputation through the graft + SD. Data from implants into different positions, both control and experimental are pooled. An analysis of variance among means resulted in a significant difference among them ($P < 0.001$).

has been shown to be rich in hyaluronate (Toole and Gross, 1971) and fibronectin (Gulati et al., 1983; Repesh et al., 1982) both of which are thought to be involved in the regulation of cell motility (Hay, 1981). In addition, preliminary results from observations of sections of early regenerates containing marked cells originating in one portion of the circumference, show that the progeny of the grafted cells become displaced from their site of origin during dedifferentiation and blastema formation (Wise, personal communication).

Our view that the eventual distribution of marked cells participating in regeneration will depend on the details of wound healing and the extent of initial cell migration during blastema formation, is not inconsistent with the apparently different results of three recent studies which have followed the fate of marked cells during regeneration. The first of these are the studies of Muneoka and Bryant (1984a,b) on the contribution of graft and host cells to supernumerary limbs formed in the developing and regenerating limbs of axolotls. In these experiments, limb buds or blastemas were grafted contralaterally to appose anterior and posterior cells of the host and graft. Cells of host and graft origin were distinguished by being either diploid or triploid. The supernumerary limbs which formed at the graft/host junction at the sites of maximum positional disparity were found to consist of approximately equal contributions of graft and host cells. Of particular interest to the results we are discussing here, is the fact that the boundary between the two cell types was relatively distinct, and evidence of extensive cell mixing during outgrowth was absent. The discrepancy between this result, and our results which indicate extensive mixing of cells during outgrowth, could be attributed to differences involved in blastema formation in the two experiments. When supernumerary limbs form from a graft junction, extensive cell movement across an open wound surface is not involved, and thus we would not expect to find cells of host and graft origin extensively intermingled.

The second study which is pertinent to consider is that of Slack (1980) who analyzed the location of triploid cells derived from a half-cuff of skin transplanted to the posterior side of a diploid axolotl forearm. Following amputation through the forearm, marked cells were largely confined to the side of the regenerate closest to the marked graft. While these results appear to be inconsistent with those reported here, it is possible that the failure of marked cells to cross from anterior to posterior during regeneration from the forearm is related to the mode of wound healing. Results from a variety of experiments (see Tank and Holder, 1981, for a review) have led to the conclusion (Bryant et al., 1981; Holder, 1981; Holder and Reynolds, 1983, 1984; Holder et al., 1980; Krasner and Bryant, 1980) that while healing across a stump in the upper arm can involve confrontation between cells from any circumferential position, healing across a stump in the lower arm leads to interactions between cells from dorsal and ventral positions. Hence, if preferential dorsal to ventral interactions reflect a dorsal to ventral migration preference in the lower arm, marked cells which originate in either the anterior or posterior of the forearm would be expected to remain confined to that region, as Slack's results show.

Finally Tank et al. (submitted) have recently studied the fate of marked cells in regenerates derived from surgically constructed axolotl upper arms in which one entire half consists of diploid tissue and the other of triploid tissue. Tank et al. found that about three fourths of the triploid cells were confined to the half
of the regenerate closest to the triploid half of the stump. Preliminary data from our laboratory indicates that about 50% of the cells of the blastema are of dermal origin (Muneoka et al., in preparation). Hence, in the Tank et al. experiments, where tissues in addition to demis were grafted and scored, approximately 50% of the marked cells are expected to be of dermal origin. The data presented here suggest that this 50% of the blastema cells will be dispersed to all parts of the final regenerate. The distribution of cells reported in the Tank et al. experiments is entirely consistent with this interpretation.

In conclusion, based on the experiments reported here, we reiterate the idea proposed earlier (Bryant et al., 1981) that distal outgrowth of the limb is a product of intercalation between cells from different parts of the limb circumference. The available evidence is consistent with the idea that the extent to which marked cells from one part of the circumference provide progeny which are distributed to different parts of the circumference of the regenerate, is dependent upon the extent of initial cell migration and mixing during wound healing and blastema formation.

The authors sincerely thank Dr. R. Campbell, Dr. D. Gardiner, Dr. R. Meyer, and Dr. K. Muneoka for their helpful comments and criticisms. We also thank Warren Fox for his comments and his membret dans le developpement des membres surnumeraires induits by PHS Grants HD 06082 and HD 07029. EmbryoL Exp. Morphol. 38, 151-173.

In conclusion, based on the experiments reported

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