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Journal

Developmental biology, 105(1)

ISSN

0012-1606

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Publication Date

1984-09-01

DOI

10.1016/0012-1606(84)90272-0

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Cellular Contribution to Supernumerary Limbs in the Axolotl, *Ambystoma mexicanum*

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Received January 6, 1984; accepted in revised form April 30, 1984

Using the triploid cell marker, the cellular contribution from graft and stump to the supernumerary limbs which result from contralateral grafts of limb buds and regeneration blastemas in the axolotl has been analyzed. Grafts were made so as to appose anterior and posterior limb positions. Overall, the contribution from graft and stump tissue was found to be approximately equal although the position of the boundary between the two was variable from limb to limb. This result is consistent with models which suggest that intercalary regeneration is the driving force for patterning of the vertebrate limb. In addition, the pattern of cellular contribution to supernumerary limbs was consistently found to be asymmetrical in the dorsal-ventral axis. Hence, posterior limb tissue predominantly contributed cells to the posterior and dorsal part of the supernumerary limb whereas anterior limb tissue predominantly contributed cells to the anterior and ventral part of the supernumerary limb. The reason for this asymmetrical pattern remains unknown, but we suggest that it might result from a directional bias in intercalary regeneration, similar to that observed during intercalation in the proximal-distal axis of the urodele limb. Using the triploid cell marker in conjunction with a black/white pigmentation marker, the relationship between the cellular contribution boundary and the pigmentation boundary in supernumerary limbs has also been analyzed. It has been found that the positions of the two boundaries do not coincide, a result which suggests that the eventual location of pigment cells is not a good indicator of the location of nonpigment cells derived from graft and stump.

INTRODUCTION

In urodeles, supernumerary limbs can result from a number of different experimental grafting manipulations of the regeneration blastema and the developing limb bud (regeneration—reviewed in Tank and Holder, 1981; development—Maden and Goodwin, 1980; Thoms and Fallon, 1980; Muneoka and Bryant, 1982). It is from such experimental manipulations that our current understanding of how the limb pattern is established or reestablished during limb outgrowth has evolved. The polar coordinate model (French *et al.*, 1976; Bryant *et al.*, 1981) has in recent years provided a framework for exploring epimorphic pattern regulation. This model proposes that cells behave as if they possess positional information (Wolpert, 1969) about both their location along the proximal-distal axis of the limb and their location around the limb circumference. The most important feature of this model is its proposal that tissues have the general property of intercalary regeneration: cell division is stimulated when normally nonadjacent cells come into contact and intermediate positional values are generated in response to a positional disparity. An alternative view proposes that positional information is specified with respect to the local concentration of a diffusible morphogen produced at a specialized region (polarizing zone) of the limb bud or blastema (Tickle *et al.*, 1975; Meinhardt, 1982, 1983; see Tank and Holder, 1981).

While both of these models can quite accurately describe the phenomena associated with pattern regulation, such as the production of supernumerary limbs following various grafting operations, not enough is known about the cellular processes involved in the formation of supernumerary limbs to allow us to differentiate between such disparate views of patterning in the vertebrate limb. An understanding of the cellular origins of supernumerary limbs is an important step toward analysis of patterning processes at the cellular level. Previous studies of the cellular contribution from graft and stump tissues to supernumerary limbs resulting from grafting experiments performed on the chick limb bud, the *Xenopus* limb bud, the axolotl limb bud, and the axolotl regeneration blastema have yielded varying results. Using the chick/quail cell marker system, Iten and co-workers have shown that cells of both graft and host origin contribute to supernumerary limb structures in the chick following 180° rotation of the limb bud (Javois and Iten, submitted; Iten *et al.*, 1983), grafts of wedges of tissue from posterior to anterior (Iten, 1982), grafts of wedges of tissue from anterior to posterior (Iten and Murphy, 1980), and grafts of wedges of tissue from dorsal to ventral and *vice versa* (Javois and Iten, 1982). However, Honig (1983) has reported that the cellular contribution to supernumerary limbs resulting from grafts of anterior wedges from quail limb buds to posterior positions in the chick wing is almost entirely of graft (i.e., anterior) origin. Working with

Xenopus larvae, Cameron and Fallon (1977) also concluded that the cellular contribution (based on the 1-nu cell marker) to supernumerary limbs resulting from 180° rotation of the hindlimb bud is solely from anterior tissue. In the developing axolotl, however, Thoms and Fallon (1980) using the triploid cell marker, showed a cellular contribution from both graft and stump to supernumerary limbs which were formed after various axial misalignments of the forelimb bud. Studies of the relative contribution from graft and stump to supernumerary limbs resulting from various types of axial misalignment of the regeneration blastema have made use of differences between black and white axolotls (Tank, 1978; Maden and Turner, 1978; Wallace and Watson, 1979) or of species-specific differences in the skeletal morphology of *A. mexicanum*, *A. maculatum*, and *A. texanum* (Stocum, 1982). Taken as a whole, these blastema studies have indicated that supernumerary limbs arise from graft tissue only, stump tissue only, or from a combination of both stump and graft tissue. However, both markers are less than ideal, and cannot be followed on a cell by cell basis in the supernumerary outgrowth (see Discussion).

Given the diversity of results from experiments on the same species, from experiments on different species, and from experiments on developing and regenerating limbs, we have undertaken a detailed analysis (using the triploid cell marker) of the cellular contribution from graft and stump tissues to supernumerary limbs produced from both contralateral blastema and limb bud grafts in the axolotl. In this paper we discuss the data from grafts to appose anterior and posterior limb bud or blastema regions with regard to (1) the various models for the formation of pattern in the vertebrate limb, (2) the consistently asymmetrical pattern of cellular contribution to supernumerary limbs revealed in this study, and (3) the relationship between the boundary formed between triploid and diploid tissues and the pigmentation boundary formed between black and white tissues within supernumerary limbs.

MATERIALS AND METHODS

All experiments were performed on axolotls (*A. mexicanum*) spawned at the University of California, Irvine. Animals were maintained at $20 \pm 1^\circ\text{C}$. Prior to grafting, young larvae were kept in pans of 20% Steinberg's solution. They were changed and fed newly hatched brine shrimp daily. Experimental animals were maintained individually in 100×25 -mm plastic petri dishes in 25% Holtfreters solution until they reached a body length of 5 cm. They were changed and fed tubifex worms daily. Animals larger than 5 cm were kept in 1-liter plastic boxes and they were changed and fed tubifex worms three times a week.

Triploid larvae were produced by subjecting fertilized eggs to hydrostatic pressure (6000 psi for 8 min) using the protocol of Gillespie and Armstrong (1979). Treated animals were screened for triploidy following the protocol outlined in Muneoka *et al.* (1984). Triploid larvae possess cells with three nucleoli while diploid larvae possess cells with two (Fankhauser and Humphrey, 1943). Triploid and diploid larvae were maintained under identical conditions but were kept separately.

Grafting

The grafting procedures used in this study are shown in Fig. 1. As shown in Fig. 1a, hindlimb buds were exchanged contralaterally between diploid and triploid axolotls so as to appose anterior and posterior limb tissues (A/P contralateral limb bud grafts). Limb buds used in these experiments were from the hindlimbs of stage 46 (Schreckenbach and Jacobson, 1975) animals ranging in body length from 29 to 35 mm. The hindlimb bud at this stage is dome-shaped, flattened dorsal-ventrally and internally undifferentiated (Muneoka and Bryant, 1982). Figure 1b shows the grafting operation for anterior-posterior apposed contralateral blastema grafts between diploid and triploid animals (A/P contralateral blastema grafts). Blastemas utilized in these experiments were at the palette stage (Tank *et al.*, 1976) and were from forelimbs of animals ranging in body length from 38 to 62 mm. All grafts were made between sibling axolotls and each host animal received a single graft. To maximize the use of the triploid larvae, the two hindlimb buds or blastemas of each triploid animal were grafted to two separate diploid hosts. One of the two diploid host larvae served as a donor for the triploid animal. When possible, a pigment marker (white (dd)/black (Dd)) was utilized in conjunction with the ploidy marker.

Animals were anesthetized in MS222 (Eastman) diluted 1:3000 in 20% Steinberg's solution. Grafting was performed in air using fine forceps, microdissection scissors, and a finely sharpened microdissection scalpel. The dorsal surface of the limb bud or blastema was labeled with carmine particles to insure accurate axial orientation of the graft. A small piece of lens paper was placed beneath the limb bud or blastema and after amputation the tissue was transferred to the contralateral host limb on the lens paper. This procedure avoided damage to the grafted tissue. The graft was placed in the appropriate orientation on the host stump and the lens paper was carefully removed. The graft was held in place with small strips of lens paper and the animal was placed on ice for 2–5 min. The animal was then carefully placed into a dish of slightly cooled (approximately 15°C) 20% Steinberg's solution. Each animal

FIG. 1. Diagram of the grafting procedures. (a) Limb buds, shown here as viewed from the ventral aspect. Hindlimb buds were exchanged contralaterally between diploid (left) and triploid (right) sibling axolotls and oriented on the host limb bud stump to appose anterior and posterior limb tissues. Dorsal and ventral limb bud tissues were positioned in correct axial alignment with the stump. The insert shows a closer lateral view of a right triploid limb bud grafted onto a left diploid limb bud stump. (b) Blastemas, shown here as viewed from the dorsal aspect. Forelimb blastemas at the stage of palette were exchanged contralaterally between diploid (left) and triploid (right) sibling axolotls and oriented on the host to appose anterior and posterior limb tissues. Dorsal and ventral blastema tissues were positioned in correct axial alignment with the stump. The inset shows a closer dorsal view of a left triploid blastema grafted onto a right diploid stump.

was observed daily until the graft was well-healed after which time observations were made 2-3 times a week.

Tissue Processing

Four to 8 weeks after grafting, when well-formed supernumerary outgrowths were apparent, limbs were

amputated, fixed in Carnoy's fixative, and processed immediately. A total of 40 supernumerary limbs were analyzed (20 supernumerary limbs from each grafting operation) to determine the cellular contribution from the stump and graft. In 10 of the supernumerary limbs resulting from limb bud grafts, the cellular contribution of the graft and stump to both the cartilage and the dermis was analyzed and in the remaining 30 supernumerary limbs only the dermis was analyzed.

Limbs in which the white/black pigment marker was used in conjunction with the triploid/diploid cell marker were drawn with the aid of a camera lucida so as to record the location of the pigment boundary. The dorsal and ventral skin (epidermis and dermis) of all limbs was removed in two separate sheets and the dermis was isolated and stained with bismuth for whole-mount dermal analysis (Muneoka *et al.*, 1984). The skeletal patterns of the skinned limbs were drawn with the aid of a camera lucida to record the number and arrangement of digits and phalangeal elements. Limbs which were to be analyzed for cellular contribution to the cartilage, were hydrated, decalcified in 10% Versene (pH 6.5), dehydrated, cleared, and embedded in paraffin. Serial longitudinal sections were cut at 10 μ m and stained with bismuth (Locke and Huie, 1977; Muneoka *et al.*, 1984). The remaining skinned limbs were processed for whole-mount analysis of the skeletal pattern by Victoria blue B staining (Bryant and Iten, 1974).

Analysis of Data

In 10 supernumerary limbs resulting from limb bud grafts, the cellular contribution to the cartilage was analyzed in bismuth-stained serial longitudinal sections through the digits of the supernumerary limb. In general, cell counts were made on every other section of the entire metatarsal element of each digit. In cases where the digit was reduced and not many scorable cells were present in the metatarsal, counts were made on more distal phalangeal structures as well. In cases where two digits articulated with a single metatarsal, counts were made only on phalangeal elements distal to the point of bifurcation. The trinucleolate cell frequency in sectioned cartilage reflects the number of cells with three nucleoli divided by the total number of cells with two and three nucleoli. Cells with zero or one nucleolus were not scored in sectioned tissue.

Cellular contribution to the dermis was analyzed in bismuth-stained dorsal and ventral whole-mount dermal preparations (Muneoka *et al.*, 1984) of the digits of all 40 supernumerary limbs. The frequency of trinucleolate cells in dermal preparations was calculated by dividing the number of trinucleolate cells in a digit by the total number of scorable cells in each digit. All cells of each

digit, with the exception of red blood cells, were scored and in cases where part of the digit dermis was damaged in processing, thus reducing the total number of cells available for counting, cells at the base of the digit were also scored. Cells were designated as unscorable if no nucleoli were evident or if cells were overlapping and there was some question about the number of nucleoli per cell.

The frequency of trinucleolate cells of each digit of a supernumerary limb was compared to its individual control frequency. In cases where triploid limb buds or blastemas were grafted to a diploid host, the control trinucleolate cell frequency was determined from cell counts of the grafted region of the limb. In cases where triploid animals served as hosts for diploid grafts, the control trinucleolate frequency was determined in each case from cell counts of the contralateral ungrafted triploid limb.

The frequency of trinucleolate cells for each digit, based on counts of the dorsal dermis, cartilage (in 10 cases), and ventral dermis, was plotted across the anterior-posterior axis of each supernumerary limb (Fig. 2). Since the transition from a high trinucleolate cell frequency to a low trinucleolate frequency tended to be rather abrupt, we considered the point where the plotted line crossed 50% of control trinucleolate counts as the boundary (50% boundary) point between triploid and diploid dermal cells. The digit or interdigital region (Fig. 2) nearest to the 50% boundary point was considered to be the boundary. A line connecting the boundary in the dorsal dermis, cartilage (in 10 cases), and ventral dermis was used to approximate the boundary between triploid and diploid tissues.

Limb Pattern

The criteria used to identify the pattern of the supernumerary limbs were those of Pescitelli and Stocum (1980). In cases where the skeletal pattern was derived from sectioned tissue, the number of phalangeal elements for each digit was determined from camera lucida drawings of skinned limbs and the number, location, and articulations of basal tarsals were reconstructed from serial sections of the limb. Limbs selected for analysis in this study were those limbs which appeared to have the most complete supernumerary limbs and were maximally expanded in the anterior-posterior plane of the limb. Limbs with a reduced number of supernumerary digits and/or supernumerary limbs which during outgrowth became twisted relative to the grafted limb were not analyzed in this study. In cases where the graft and the supernumerary limb shared a digit on the line of symmetry between them (e.g., graft plus supernumerary limb digit sequence of 1, 2, 3-4-3, 2, 1), the shared

FIG. 2. Histograms showing the frequency of trinucleolate cells in the dermis and cartilage of each digit of a supernumerary limb. In each histogram the control (C) frequency is shown on the far right (solid column). In this example, a five digit posterior supernumerary limb (Fig. 5 and Table I, limb No. 5) resulted from a diploid limb bud grafted onto a triploid limb bud stump. The frequency of trinucleolate cells in the dorsal dermis (top), cartilage (middle), and ventral dermis (bottom) was similar to the control frequency in digits three, four, and five (cross-hatched columns) indicating that the cells of these three digits originated predominately from the triploid stump. Digits one and two on the other hand, had a very low frequency of trinucleolate cells indicating that they arose predominately from the diploid grafted limb bud cells. The approximate position of the boundary between triploid and diploid cells for each of these three preparations (dorsal dermis, cartilage, ventral dermis) was estimated as the point where the line connecting the trinucleolate frequency crossed 50% of the control trinucleolate frequency. That position is indicated by an arrow in each histogram and is shown diagrammatically in an idealized representation of a transverse section through the digital region of the supernumerary limb (lower diagram). In this example, the position of the boundary between triploid and diploid cells was between digits two and three in the dorsal dermis, cartilage, and the ventral dermis (solid circles). The line connecting the position of the boundary in the three preparations represents the overall cellular contribution from stump versus graft to the supernumerary limb. The bar above each frequency represents the upper 95% confidence limit.

digit was scored as part of the supernumerary limb pattern for purposes of this analysis.

RESULTS

Digital Patterns of Supernumerary Limbs

The patterns of 20 experimental limbs, each consisting of the grafted limb plus two supernumerary limbs, were analyzed in this study. The skeletal patterns of all experimental limbs resulting from both limb bud and blastema grafting were similar. Supernumerary limbs

were found to form at anterior and posterior locations relative to the stump (Fig. 3). All 40 supernumerary limbs were of stump handedness and were fused with the grafted limb, which retained its original donor handedness. Supernumerary limbs generally branched from the grafted limb in the autopodial region and the three limbs together formed an expanded array of digits (Fig. 3).

The majority (29/40) of the supernumerary limbs were classified as complete supernumerary limbs, i.e., they possessed four digits (17/20 forelimb blastema grafts) or five digits (12/20 hindlimb bud grafts). The remaining supernumerary limbs (11/40) were classified as incomplete, possessing less than four digits (3/20 forelimb blastema grafts) or less than five digits (8/20 hindlimb bud grafts). Almost all incomplete supernumerary limbs (10/11) formed anterior (with respect to the stump) to the grafted limb. Most supernumerary limb digital patterns (37/40) were continuous with respect to the grafted limb digital pattern and were arranged in mirror symmetry with the grafted limb. For example, the limb in Fig. 3 shows a digital pattern of 1, 2, 3, 4 (anterior supernumerary limb)—3, 2, 1 (grafted limb)—1, 2, 3, 4 (posterior supernumerary limb). Note that both super-

numerary limbs formed mirror symmetrical patterns with the grafted limb pattern. Even in cases where the supernumerary limb was incomplete, the grafted limb was also found to be incomplete, again forming a mirror symmetrical pattern with the supernumerary limb, e.g., digital pattern 1, 2, 3 (supernumerary limb)—3, 2, 1 (grafted limb). Of the three cases with discontinuous digital patterns, two resulted from limb bud grafts and occurred on the same experimental limb, forming an overall digital sequence of 1, 2, 3 (anterior supernumerary limb)—5, 4, 3, 2, 1 (grafted limb)—3, 4, 5 (posterior supernumerary limb). The remaining case resulted from a blastema graft and formed a three digit anterior supernumerary limb with a digital sequence of 1, 3, 4.

Cellular Contribution to Supernumerary Limbs

Control cell counts. The frequency of trinucleolate cells calculated from counts of control triploid tissue varied considerably from animal to animal, but not from limb to limb within a single triploid animal (see Muneoka *et al.*, 1984). In this study the frequency of trinucleolate cells derived from counts of whole-mount dermal preparations in different triploid animals varied from 0.31 to 0.76 while the frequency derived from counts of cartilage in sections varied from 0.37 to 0.49. The frequency of trinucleolate cells in whole-mount dermal preparations and sectioned cartilage of diploid animals was 0.00 and 0.03, respectively (Muneoka *et al.*, 1984). An example of a dermal preparations showing binucleolate and trinucleolate dermal cells is shown in Fig. 4. Binucleolate and trinucleolate cartilage cells in paraffin-sectioned tissue are also shown in Fig. 4.

Experimental limb bud grafts. An analysis was made of the cellular contribution from graft and stump to both the dermis and cartilage of 10 supernumerary limbs resulting from limb bud grafts. This was achieved by analyzing both whole-mount dermal preparations and serially sectioned cartilage of the same limb. The results of this analysis are shown in Fig. 5 and Table 1. In all 10 limbs, a cellular contribution from both stump and graft was observed in the dermis and the cartilage. The extent of the contribution from the stump versus that from the graft was found to vary from limb to limb. However, in all 10 of these supernumerary limbs the contribution boundary within the cartilage fell on or very close to the line connecting the dorsal and ventral diploid/triploid boundaries in the dermis (Fig. 5). These data suggest that the analysis of the cellular contribution to the dermis of supernumerary limbs resulting from experimental grafts of this type represents a reasonable approximation of the cellular contribution to more internal tissues such as the cartilage. Hence, in the remaining supernumerary limbs in this study, dermis alone was analyzed (Fig. 6 and Table 1).

FIG. 3. Skeletal preparation of an experimental limb as viewed from the dorsal aspect. This limb (Fig. 7 and Table II, limb No. 12) resulted from a graft of a left triploid blastema onto a right diploid stump. Two supernumerary limbs formed; one anterior (A) and the other posterior (P) to the grafted limb. The grafted limb maintained its original handedness and both supernumerary limbs are of stump handedness. The overall digital sequence from anterior to posterior (left to right) is 1, 2, 3, 4 (anterior supernumerary limb), 3, 2, 1 (grafted limb), 1, 2, 3, 4 (posterior supernumerary limb). $\times 7.2$.

FIG. 4. (a) Whole-mount dermal preparation. The dermis was manually isolated from the epidermis and analyzed in bismuth-stained whole mounts for cellular contribution to supernumerary limbs. An example of a dorsal dermal preparation is shown here. Epidermis is left at the digit tips to prevent the dermal preparation from curling. The limb from which this preparation was derived formed supernumerary limbs anterior (left 4 digits) and posterior (right 4 digits) to the graft (central 3 digits). $\times 7$. (b, c) A higher magnification of bismuth-stained triploid (b) and diploid (c) regions in the dermis of a supernumerary limb. (d, e) Examples of trinucleolate (d) and binucleolate (e) cartilage cells in paraffin sections stained with bismuth.

FIG. 5. Summary diagram of the cellular contribution boundaries in the dorsal dermis, cartilage, and ventral dermis of 10 supernumerary limbs resulting from contralateral limb bud grafts. Five supernumerary limbs formed anterior to the graft (left column) and five formed posterior to the graft (right column). The experimental limbs which gave rise to these supernumerary limbs are numbered 1-5. The boundary position in the dorsal and ventral dermis and in the cartilage of each supernumerary limb was derived from data similar to those shown in Fig. 2. These data are presented for each individual supernumerary limb in Table 1. Complete supernumerary limbs are those with five digits and are shown diagrammatically as full ellipses. Incomplete supernumerary limbs possess less than five digits and are shown as partial ellipses. Cross hatching indicates triploid tissue.

In the remaining 10 cases derived from limb bud grafts, both the stump and the graft contributed to the dermis of the supernumerary limbs. The amount of contribution from the graft versus that from the stump, and the position of the boundary again varied from limb to limb as was the case for the 10 limbs discussed above. Considering all 20 supernumerary limbs, the dorsal and ventral boundaries within each individual limb, for the most part, did not fall at the same position on the anterior-posterior axis of the supernumerary limb. There was a tendency for the dorsal boundary to be closer to the anterior edge of the limb, and for the ventral boundary to be closer to the posterior edge. This indicates that cells derived from the posterior edge of the stump or graft tend to contribute more to the dorsal than to the ventral part of the supernumerary limb while cells from the anterior edge of the stump or graft tend to contribute more to the ventral than to the dorsal part of the supernumerary limb.

The nucleoli stain dark brown and are easily distinguished with this staining procedure. In diploid tissue cells have nuclei with two nucleoli while in triploid tissue, cells have nuclei with three nucleoli. $\times 612$.

TABLE 1

Limb No. (donor/host)	Anterior					Posterior				
	1	2	3	4	5	1	2	3	4	5
1										
D	0.01	0.37	0.77	0.75	—	0.72	0.58	0.21	0.00	0.00
C	0.00	0.00	0.36	0.56	—	0.51	0.60	0.26	0.01	0.00
V	0.00	0.01	0.05	0.74	—	0.68	0.61	0.68	0.52	0.01
2										
D	0.20	0.49	0.55	0.54	0.54	0.40	0.17	0.08	0.02	0.01
C	0.05	0.29	0.31	0.35	0.36	0.32	0.19	0.09	0.01	0.01
V	0.30	0.24	0.40	0.46	0.44	0.53	0.47	0.48	0.18	0.00
3										
D	0.03	0.47	0.47	0.49	—	0.42	0.04	0.00	0.00	0.00
C	0.00	0.23	0.36	0.39	—	0.35	0.36	0.11	0.00	0.00
V	0.00	0.10	0.39	0.51	—	0.54	0.55	0.47	0.17	0.00
4										
D	0.32	0.46	0.52	0.57	—	0.51	0.12	0.01	0.00	0.00
C	0.00	0.10	0.47	0.41	—	0.41	0.41	0.21	0.02	0.00
V	0.00	0.02	0.22	0.43	—	0.46	0.49	0.36	0.12	0.00
5										
D	0.52	0.08	0.06	—	—	0.02	0.08	0.66	0.72	0.74
C	0.32	0.39	0.02	—	—	0.00	0.01	0.38	0.39	0.34
V	0.52	0.12	0.02	—	—	0.00	0.02	0.54	0.64	0.68
6										
D	0.03	0.36	0.64	0.60	0.66	0.61	0.28	0.04	0.00	0.00
V	0.00	0.02	0.11	0.09	0.47	0.61	0.65	0.59	0.40	0.00
7										
D	0.07	0.46	0.63	0.59	—	0.27	0.00	0.02	0.01	0.00
V	0.03	0.25	0.57	0.63	—	0.59	0.58	0.54	0.09	0.05
8										
D	0.79	0.51	0.07	0.02	—	0.02	0.20	0.77	0.85	0.82
V	0.78	0.78	0.65	0.01	—	0.00	0.01	0.52	0.70	0.76
9										
D	0.04	0.25	0.30	0.29	0.30	0.22	0.06	0.02	0.01	0.00
V	0.00	0.08	0.26	0.27	0.24	0.28	0.26	0.14	0.01	0.00
10										
D	0.00	0.42	0.43	—	—	—	—	0.33	0.00	0.00
V	0.00	0.00	0.11	—	—	—	—	0.52	0.29	0.00
Experimental dermis Experimental cartilage Control dermis Control cartilage										
Total cells scored	28,825	14,664	12,492	6104						
Mean	324/Digit	326/Digit	1249/Limb	1221/Limb						
Range	186-682/Digit	157-581/Digit	913-1,605/Limb	668-2,267/Limb						

Note. The data presented in this table were used to prepare Figs. 5 and 6. The experimental limbs analyzed are listed in the far left column (limbs) along with the ploidy of the graft and the stump for that individual limb. The far right column lists the control trinucleolate frequency for each individual preparation. In the cases where the graft was triploid, the control trinucleolate frequency was calculated from cell counts of the mature graft itself. In cases where the host was triploid, the control frequency was from cell counts of the contralateral limb and this frequency was used as a control for both dorsal and ventral dermal preparations. The frequency of trinucleolate cells is listed for the dorsal dermis (D), cartilage (C), and ventral dermis (V) of each supernumerary digit. Horizontal bars indicate the absence of that supernumerary digit. The bottom of the table lists the total number of cells scored, the mean number of cells scored, and the range of number of cells scored in the experimental and control limbs in this study.

FIG. 6. Summary of the cellular contribution from the stump and graft to 10 supernumerary limbs resulting from contralateral limb bud grafts in which only the dorsal and ventral dermis was analyzed. Five anterior (left column) and five posterior (right column) supernumerary limbs are shown as in Fig. 5. See legend to Fig. 5 for details.

In most cases the boundary between triploid and diploid cells was observed to be relatively sharp, that is, the abundance of trinucleolate cells dropped abruptly from one region to the next. In a few cases however, the frequency of trinucleolate cells decreased gradually along the anterior-posterior limb axis suggesting a rather diffuse boundary between marked and unmarked cells. Furthermore, there were cases in which the dorsal and ventral dermal boundaries of the same supernumerary limb were different in terms of the sharpness of the boundary (e.g., dorsal sharp; ventral diffuse).

Experimental blastema grafts. The cellular contribution from graft and stump to 20 supernumerary limbs was analyzed in dorsal and ventral whole-mount dermal preparations. As was the case in the limb bud grafts, all 20 of the supernumerary limbs analyzed in this study showed a substantial cellular contribution from both the graft and the stump (Fig. 7; Table 2). In individual cases, the actual amount of contribution from either stump or graft varied, but overall the amount was approximately equal. The position of the boundary tended to fall in the region of digits two and three but was not constant from limb to limb. As in the limb bud grafts, the position of the boundary in the dorsal dermis never coincided with the boundary in the ventral dermis. The position of the dorsal boundary line was always closer to the anterior edge of the supernumerary limb whereas that of the ventral boundary line was always closer to the posterior edge of the supernumerary limb. This distinctive asymmetric pattern of cellular contribution to

supernumerary limbs was observed in all 20 supernumerary limbs derived from blastemas grafts and in 17/20 supernumerary limbs derived from limb bud grafts. In a separate experiment, the same asymmetrical cellular contribution was observed in 15/20 supernumerary limbs resulting from contralateral grafts between limb buds and blastemas (Muneoka and Bryant, 1984). In all of these experimental situations, the remaining supernumerary limbs (8/60) showed a symmetrical dorsal-ventral pattern of cellular contribution, where posterior and anterior cells contributed equally to dorsal and ventral parts of the supernumerary limbs. In no case did a supernumerary limb show a pattern of cellular contribution which had the opposite asymmetry to that of the majority.

Pigmentation versus Ploidy Boundaries

Twenty-eight of the 40 supernumerary limbs analyzed in this study resulted from grafts in which the donor and host differed not only in terms of their ploidy, but

FIG. 7. Summary diagrams of the cellular contribution boundaries in the dorsal dermis and ventral dermis of 20 supernumerary limbs formed from A/P contralateral blastema grafts. Ten supernumerary limbs formed anterior to the graft and 10 formed posterior to the graft. The experimental limbs which gave rise to these supernumerary limbs are numbered 11-20. The data for each individual supernumerary limb is presented in Table 2. See legend to Fig. 5 for details.

TABLE 2

Limb No. (donor/host)		Anterior				Posterior				Control
		1	2	3	4	1	2	3	4	
11	D	0.23	0.08	0.03	0.02	0.15	0.59	0.66	0.67	0.66
(2N/3N)	V	0.62	0.66	0.54	0.06	0.02	0.04	0.28	0.71	
12	D	0.01	0.47	0.59	0.62	0.61	0.19	0.02	0.00	0.61
(3N/2N)	V	0.01	0.00	0.03	0.68	0.73	0.62	0.13	0.20	0.68
13	D	0.09	0.56	0.66	—	0.62	0.07	0.03	0.01	0.60
(3N/2N)	V	0.01	0.07	0.45	—	0.55	0.55	0.48	0.20	0.62
14	D	0.00	0.40	0.64	0.67	0.68	0.39	0.01	0.01	0.67
(3N/2N)	V	0.01	0.00	0.15	0.71	0.72	0.77	0.53	0.02	0.68
15	D	0.05	0.26	0.36	—	0.33	0.11	0.02	0.02	0.32
(3N/2N)	V	0.00	0.01	0.25	—	0.41	0.41	0.15	0.02	0.39
16	D	0.45	0.07	0.04	0.02	0.01	0.45	0.56	0.59	0.63
(2N/3N)	V	0.62	0.61	0.38	0.02	0.02	0.03	0.26	0.59	
17	D	0.19	0.64	0.65	0.69	0.40	0.07	0.01	0.00	0.69
(3N/2N)	V	0.00	0.03	0.32	0.73	0.67	0.72	0.66	0.23	0.69
18	D	0.44	0.06	0.04	0.05	0.06	0.58	0.62	0.64	0.67
(2N/3N)	V	0.75	0.73	0.44	0.02	0.01	0.02	0.05	0.55	
19	D	0.41	0.09	0.06	0.01	0.01	0.11	0.34	0.41	0.38
(2N/3N)	V	0.37	0.30	0.05	0.03	0.03	0.03	0.07	0.39	
20	D	0.48	—	0.17	0.02	0.00	0.31	0.63	0.65	0.67
(2N/3N)	V	0.65	—	0.64	0.05	0.04	0.06	0.33	0.64	
Experimental dermis					Control dermis					
Total cells scored					48,308					14,806
Mean					627/Digit					1481/Limb
Range					304-1338/Digit					750-2527/Limb

Note. The data presented in this table were used to prepare Fig. 7. See the legend of Table 1 for details.

also in pigmentation. Since pigment cells are not abundant ventrally, comparisons between the pigmentation and ploidy boundaries were restricted to the dorsal dermis. The results of these comparisons are presented in Fig. 8. Also included in Fig. 8 are the results of comparisons between the ploidy and pigmentation boundaries in supernumerary limbs derived from a separate experiment in which contralateral grafts were made between blastemas and limb buds (Muneoka and Bryant, 1984). In this analysis if the pigmentation and ploidy boundaries coincided, all points would fall on the line drawn. In fact the coincidence between the ploidy and pigmentation boundaries is poor, suggesting that the location of pigment cells is not an accurate reflection of the location of other cell types of similar (i.e., graft or stump) origin. The supernumerary limbs plotted here ($n = 44$) resulted both from grafts where the stump was pigmented and the graft unpigmented (41%) and from grafts where the graft was pigmented and the stump unpigmented (59%). With only few exceptions, the pigmentation boundary of both posterior and anterior su-

pernumerary limbs either coincided with the ploidy boundary or fell on the grafted limb side (or distal relative to the host limb) of the ploidy boundary, regardless of whether the graft or stump was pigmented (see Fig. 8). These data suggest that pigment cells tend to migrate in a distal direction in grafting experiments of this nature.

DISCUSSION

Origin of Cells

Contralateral grafting of developing limb buds to appose anterior and posterior tissue in the axolotl (Maden and Goodwin, 1980; Thoms and Fallon, 1980; Muneoka and Bryant, 1982), *Xenopus* (Maden, 1981), *Rana* (Maden, 1981), and the chick (Saunders *et al.*, 1958) results in a high frequency of supernumerary limbs. Similarly, when blastemas are grafted to the contralateral limb stump to appose anterior and posterior limb tissues, supernumerary limbs are also formed at a high frequency (Iten and Bryant, 1975; Tank, 1978; Maden, 1980a; Sto-

FIG. 8. Graph to show the anterior-posterior position of the ploidy boundary relative to that of the pigmentation boundary in dorsal dermal preparations. In addition to the data from the limbs analyzed in this paper, data from contralateral grafts between blastemas and limb buds reported in Muneoka and Bryant (1984) are also included. Points on the line represent cases where the two boundaries are coincident. Points above the line represent cases where the pigmentation boundary is posterior to the ploidy boundary. Points below the line represent cases where the pigmentation boundary is anterior to the ploidy boundary. Anterior (A) and posterior (P) supernumerary limbs are indicated.

cum, 1982). Regardless of whether developing or regenerating limbs are used in such contralateral grafts, supernumerary limbs arise from the graft-stump junction at anterior and posterior positions and are consistently of stump handedness as predicted by the polar coordinate model (French *et al.*, 1976; Bryant *et al.*, 1981). A clear feature of our results is that in the axolotl, supernumerary limbs resulting from A/P contralateral grafts of blastemas or hindlimb buds are composed of cells from both the stump and the graft. This result is consistent with results of similar experiments on the forelimb bud of the axolotl (Thoms and Fallon, 1980), but is not in agreement with the results of Stocum (1982). After A/P contralateral blastema grafts in the axolotl, Stocum (1982) concluded that the majority of the supernumerary limbs consisted of cells from either the graft or the stump. However, in Stocum's experiment a detailed cell by cell analysis was not possible, and conclusions about the degree of contribution were based on species-specific skeletal morphology.

Analysis of the extent of cellular contribution from the stump and the graft to the supernumerary limbs formed in this study showed that although the contri-

bution boundary is occasionally diffuse, it is normally sharp, indicating that cell mixing may but need not occur during the formation of supernumerary limbs. Furthermore, our results show that both stump and graft contributed cells in approximately equal amounts. This latter result suggests that limb bud and blastema cells of the stump-graft junction respond to axial misalignment in an equivalent and mutual fashion. This interpretation is consistent with the results of grafting experiments where the cellular contribution from either the stump or the graft to supernumerary limbs resulting from A/P contralateral blastema grafts was inhibited by irradiation (Holder *et al.*, 1979). Under these circumstances, some complete supernumerary limbs formed, indicating that either anterior or posterior tissue of stump or graft origin was capable of forming the entire supernumerary limb pattern.

A striking aspect of the supernumerary limbs analyzed in this study (as well as those in Muneoka and Bryant, 1984) is the asymmetry in the cellular contribution to dorsal and ventral parts of the supernumerary limbs (Fig. 9). There is clearly a tendency for posterior cells to contribute more to the dorsal part of the supernumerary limb and for anterior cells to contribute more to the ventral part. In trying to understand this very common phenomenon three possibilities stand out,

FIG. 9. The cellular contribution boundaries derived from dermal analysis of supernumerary limbs resulting from anterior-posterior contralateral grafts of blastemas (top), limb buds (bottom), and between blastemas and limb buds (center) (data from Muneoka and Bryant, 1984) are superimposed onto summary diagrams. Each diagram represents 20 supernumerary limbs. There was substantial cellular contribution from both graft and stump to all 60 supernumerary limbs analyzed. The location of the boundary is variable from limb to limb and tends to show an asymmetry with posterior tissue contributing predominately to posterior and dorsal regions of the supernumerary limb and anterior tissue contributing predominantly to anterior and ventral regions of the supernumerary limb.

given the fact that there was equal cellular contribution from the stump and graft to these supernumerary limbs.

The first possibility is that during distal outgrowth and morphogenesis, the tissues of the supernumerary limb become characteristically rearranged to yield the asymmetrical contribution pattern observed. Little is known about cellular movements during outgrowth of the vertebrate limb, but what is known argues against the occurrence of any such rearrangements (see Iten, 1982; Javois and Iten, 1982; Tank *et al.*, 1984). The second possibility is that the observed asymmetry in contribution may be a consistent artifact of the grafting procedures used in these experiments. If the maximum positional disparity following grafting was not located precisely at anterior and posterior positions but at anterior-dorsal and posterior-ventral positions, and cellular contribution from the graft and stump was equal then the contribution pattern to any resulting supernumerary limbs would appear to be asymmetrical. This possibility seems unlikely since frequent observations following grafting operations indicated that supernumerary limbs form at anterior and posterior positions and not at anterior-dorsal and posterior-ventral positions, as would be expected if this idea was correct.

A third possible explanation for this asymmetrical pattern of cellular contribution is that in our experiments there was a tendency for posterior tissue to preferentially intercalate dorsal positional values and for anterior tissue to preferentially intercalate ventral positional values. In other words, there may be a preferred direction to intercalary regeneration around the circumference of the limb (in this case posterior to dorsal and anterior to ventral). This idea is attractive since asymmetrical or one-sided cellular contribution has been noted during proximal-distal intercalary regeneration in the axolotl (Stocum, 1975; Pescitelli and Stocum, 1980). In the proximal-distal limb axis cells tend to react to positional disparities in a one-sided manner; proximal cells divide to correct any positional discontinuity whereas distal cells do not appear to contribute to the intercalary growth. Our cell marker data represent the first suggestion that this type of directional preference for cellular interactions may also be occurring in the limb circumference. Further evidence for directional cellular interactions within the blastema or limb bud comes from the frequent observation of blastema or limb field derotation following 180° ipsilateral grafting operations (Blastema—Bryant and Iten, 1976; Maden and Turner, 1978; Maden, 1980a, 1982; Maden and Mustafa, 1982; Tank, 1981; Stock *et al.*, 1980; Turner, 1981; Limb field—see Nicholas, 1924). Although we offer no mechanism by which this phenomenon may be explained, it seems logical that for an entire graft to rotate (relative to the stump) in a directional manner, the cellular pro-

cesses and interactions involved (whether they be cellular movements, adhesive differences, or intercalary growth) must occur in a directional manner. Graft derotation is not observed following contralateral grafts possibly indicating that interactions between right and left limbs occur in opposite directions, thus stabilizing the position of the graft and resulting in an asymmetrical pattern of cellular contribution to supernumerary limbs as observed in our studies.

Dermal Analysis

In most studies of limb development and regeneration interpretations of the limb pattern stem almost exclusively from the analysis of skeletal morphology, and it is important that analyses of cellular contribution can be related to analyses of the skeletal pattern. We have found that the position of the line connecting the contribution boundary of the dorsal and ventral dermis closely approximates the actual boundary between stump- and graft-derived cells as determined by analysis of the internal cartilage in sections. Thus, the technique of analyzing cellular contributions in whole-mount dermal preparations appears to be an accurate and efficient method for analyzing the overall cellular contribution to supernumerary limbs. The direct relevance of dermal analysis is further emphasized by the accumulating evidence that positional information in urodele limbs appears to be primarily a property of the connective tissue, particularly that of the dermis (Bryant, 1978; Tank, 1979).

Pigmentation as a Marker

It has recently been suggested, based on studies in which the triploid cell marker has been used in conjunction with the black/white pigmentation marker in the axolotl (Slack, 1983) that these two markers mimic each other in cellular contribution experiments. The results of our analysis where the cellular boundary and the pigment boundary in the dermis are compared suggest the opposite: that there is tremendous variability between the two boundaries and that the pigment boundary cannot be used to determine the cellular contribution of surrounding cells and *vice versa*. This is an important point since a number of studies have used the black/white pigmentation marker to analyze the cellular origin of the surrounding tissue (Maden, 1979, 1980b; Maden and Turner, 1978; Maden and Wallace, 1975; Wallace *et al.*, 1974; Wallace and Wallace, 1973; Wallace and Watson, 1979; Tank, 1978; Stocum, 1982). Keller *et al.* (1982) have recently shown that in the axolotl embryo the migration of pigment cells in chimeric recombinations of white (dd) and black (Dd) ectoderm and mesoderm is controlled solely by the ectoderm. It seems

likely that the same is true for pigment cell migration in the developing and regenerating limb of the axolotl, and that the use of pigment cells as a marker reflects only the extent of the epidermal cell movements. Observations of the cellular contribution boundaries of the dermis and the epidermis to supernumerary limb structures using the triploid cell marker in the axolotl suggest that these two boundaries are not coincident (Thoms and Fallon, 1980; Slack, 1983; Tank *et al.*, 1984; Muneoka, personal observation).

Pattern Convergence

A consistent feature of studies on supernumerary limbs is the finding that not all are complete and well-separated from the limb derived from the graft. In the limbs analyzed here, and in those analyzed in a separate experiment in which contralateral grafts were made between developing and regenerating limbs (Muneoka and Bryant, 1984), 25% of the supernumerary limbs were incomplete, i.e., they possessed less than the normal number of digits. It is characteristic of these incomplete supernumerary limbs that the missing digits are those along the line of symmetry between the supernumerary limb and the graft. Two hypotheses have been put forth to explain the formation of incomplete supernumerary limbs. The first suggests that they could result from partial resorption of the graft followed by intercalary regeneration between the partial graft and the stump (Bryant *et al.*, 1981; Stocum, 1982). The second hypothesis suggests that the grafted limb and the supernumerary limb fuse during distal outgrowth and that interactions along the line of symmetry between the two limbs could lead to pattern convergence and loss of midline structures (Bryant *et al.*, 1981). Note that these two explanations are not mutually exclusive and that the final limb pattern in either case would be an incomplete supernumerary limb and also an incomplete grafted limb. In our results, almost all of the incomplete supernumerary limbs (14/15) formed anterior to the grafted limb bud or blastema, thus suggesting a difference between posterior and anterior supernumerary limbs. Application of these two hypotheses to our results would indicate that it is the posterior cells of the grafted limb bud or blastema (rather than the anterior cells) that are more sensitive to graft resorption and/or pattern convergence during distal outgrowth (Bryant *et al.*, 1981; Stocum, 1982). Differentiation in the axolotl limb during development and regeneration occurs from anterior to posterior, hence, this sensitivity is coincident with the lesser degree of differentiation of the grafted posterior limb cells. Similarly, Tank (1978) has observed that the posterior-most digit of the graft and the supernumerary limb are often fused in experiments involving contra-

lateral blastemal grafts. These observations suggest that the degree of differentiation of grafted cells may be important in understanding the details of the pattern regulatory response.

The fact that the pattern of cellular contribution to supernumerary limbs resulting from both blastema and limb bud exchanges is virtually identical lends support to the hypothesis that the mechanisms governing pattern formation during outgrowth of the limb during development and regeneration are similar (Muneoka and Bryant, 1982). Further support for this idea comes from a separate study in which the cellular contribution to supernumerary limbs resulting from A/P contralateral exchanges between limb buds and blastemas (Muneoka and Bryant, 1984) was also found to be identical to that reported here. We would argue further that with respect to particular limb patterning models, our results provide strong evidence for the occurrence of mutual and reciprocal cellular interactions between stump and graft cells during supernumerary limb formation and support the concept that intercalary regeneration is the driving force for pattern regulation (French *et al.*, 1976; Bryant *et al.*, 1981). It is not clear how polarizing zone models can explain these data. In fact, our data do not support the prediction of Meinhardt (1982; 1983) that cellular contribution to supernumerary limbs resulting from A/P contralateral grafts would either be only from the graft or only from the stump.

We thank Peter Bryant, David Gardiner, Lorette Javois, Christine Rollman-Dinsmore, Tim Sliter, and L. David Wise for helpful discussions during the course of this work. We also thank Warren Fox and Gregory Holler for technical assistance. This work was supported by grants from the National Institutes of Health, HD 07029 and HD 06082.

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