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A quantitative analysis of regeneration from chimaeric limb stumps in the axolotl

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SUMMARY

We have analysed the cellular contribution and cellular displacement which occur during regeneration from chimaeric (half triploid, half diploid) lower arms in the axolotl. In general both anterior and posterior halves contribute approximately 50 % of the regenerated limb cells. Deviations from equal contribution were observed only when anterior tissue was grafted, suggesting that anterior tissue is more sensitive to grafting operations. Approximately 25 % of all cells in the regenerated limb were found to be displaced to the opposite side of the limb. Cellular displacement was not random; 63 % of all displaced cells were found in regions adjacent to the tissue of origin.

INTRODUCTION

It has been known for many years that the limb regeneration blastema of urodeles is composed of cells of local stump origin. Recent advances for using the triploid cell marker in the axolotl, *Ambystoma mexicanum*, now make it possible to begin to analyse cellular contribution to the regeneration process in greater detail (Muneoka, Wise, Fox & Bryant, 1984). Interest in this area of research has been stimulated primarily by the proposition that local cellular interactions between cells from different positions in the limb circumference result in growth and intercalation of new cells which take on positional values appropriate for the replacement of the amputated limb (French, Bryant & Bryant, 1976; Bryant, French & Bryant, 1981). We have recently demonstrated, based on an analysis of cellular contribution, that the development of supernumerary limbs in the axolotl can be accounted for by an intercalation hypothesis (Muneoka & Bryant, 1948*a*,*b*).

Very little is known about the relative cellular contribution to the normal regenerate from different regions or different tissues of the limb stump. Tank, Connelly & Bookstein (1985) have used the triploid cell marker to show that during limb regeneration from half triploid and half diploid upper arm stumps, approximately 75 % of the observed trinucleolate cells were located on the side of the regenerated limb corresponding to the location of the triploid graft, whereas

Key words: limb regeneration, cellular contribution, pattern formation, axolotl, chimaeras, regeneration.

the remaining cells were located on the opposite side. In addition, Rollman-Dinsmore & Bryant (1984) demonstrated that the progeny of marked triploid cells from small grafts of skin made to particular positions around the circumference of the stump can be found distributed throughout the entire circumference of the regenerate. The significance of such cellular displacements during limb regeneration remains uncertain, but these observations are consistent with the proposition that some cell mixing is important to normal limb regeneration, and that the process is driven by position-specific cellular interactions leading to intercalary growth (Bryant, French & Bryant, 1981; Rollman-Dinsmore & Bryant, 1984).

While the studies of Tank *et al.* (1985) and Rollman-Dinsmore & Bryant (1984) describe the distribution of grafted cells during limb regeneration, they provide no comparable data on the contribution and distribution of cells from host tissues. Information of this type is needed since many of the conclusions from tissue grafting experiments are in fact based on the assumption that grafted tissues behave and contribute cells to the regenerate in a manner analogous to that of ungrafted tissues. In this paper we have analysed limbs regenerated from lower arm stumps consisting of half triploid and half diploid tissues. Our results demonstrate that in the majority of cases both grafted and ungrafted tissues each contribute approximately 50 % of the total cells in the regenerate. Furthermore, we provide data which are consistent with the observations of Tank *et al.* (1985) and Rollman-Dinsmore & Bryant (1984) that approximately 25 % of both grafted and host cells in the regenerated limb are found on the side opposite to that of their side of origin.

MATERIALS AND METHODS

All operations were performed on axolotls spawned at the University of California, Irvine. Animals ranged from 9·7 to 13·0 cm in length. Triploid animals were made and screened as previously described (Muneoka *et al.* 1984). All experimental animals were maintained individually in 20 % Holtfreters solution and were changed and fed tubifex worms three times a week.

The grafting operation is illustrated in Fig. 1. Triploid and diploid sibling axolotls of similar sizes were anaesthetized in MS222 and were operated on simultaneously. All operations were performed on the lower arm, exchanging either the anterior or posterior half of the limb between triploid and diploid animals. Proximal–distal cuts were made with a scalpel between the radius and ulna extending distally between digits **2** and **3**. The half limb to be grafted was then cut transversely just distal to the elbow and reciprocally exchanged with the corresponding half limb of the sibling animal. Grafts were sutured into place with 8·0 silk suture and amputated immediately. The hindlimb of each donor triploid animal was also amputated and the regenerated limb was used as a control to determine the frequency of trinucleolate cells for each individual triploid donor. Following the operation, experimental animals were observed daily to ensure that the graft had taken.

Regenerated experimental limbs as well as ungrafted but regenerated control triploid limbs were harvested at the stage of late digits (Tank, Carlson & Connelly, 1976) and fixed in Carnoy's fixative. All limbs were skinned and the skin was processed for whole-mount dermal analysis (Muneoka *et al.* 1984). The remaining skinless limbs were sectioned and stained with bismuth to determine the skeletal pattern of the regenerate. Cell counts were performed on the digits of bismuth-stained dermal whole mounts to determine the extent of cellular contribution to the

dermis of the regenerate from diploid and triploid tissue. The cellular contribution to internal tissues of the limb was not analysed in detail here since it has previously been shown to coincide with the cellular contribution to the dermis (Muneoka & Bryant, 1984*a*). Nevertheless, bismuth-stained sections of the regenerate were scanned, and we have confirmed our previous finding that dermal analyses are an accurate reflection of the cellular contribution to internal limb tissues.

Cell counts of the dorsal and ventral dermis of each digit of an experimental limb gave a trinucleolate frequency which was adjusted by comparison to that of the corresponding control triploid limb, to yield the real frequency of triploid and diploid cell contribution to each chimaeric regenerate. These real frequencies were used to determine the extent of cellular contribution from anterior and posterior tissues, as well as the extent of cellular displacement which occurred during regeneration.

RESULTS

A total of 13 limbs was analysed in this study; six limbs received anterior grafts (three cases of triploid grafts to diploid hosts; three cases of diploid grafts to triploid hosts) and seven limbs received posterior grafts (four cases of triploid grafts to diploid hosts; three cases of diploid grafts to triploid hosts). Macroscopic observation showed that all limbs regenerated normally. Serial reconstruction of

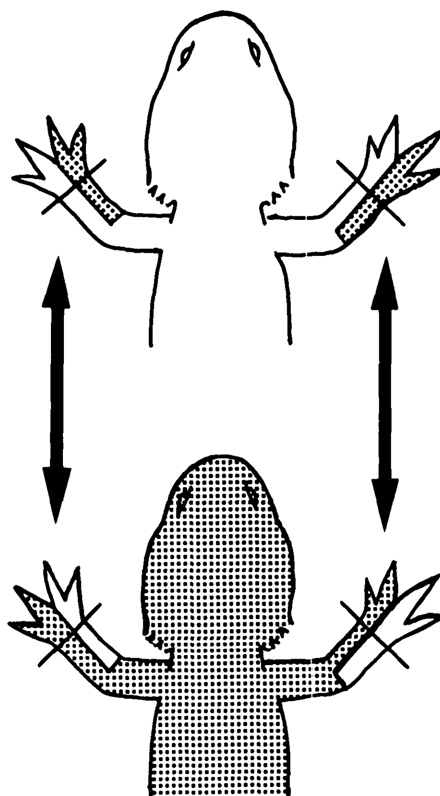

Fig. 1. Diagram of the grafting procedure as viewed from the dorsal aspect. Anterior or posterior lower limb halves were exchanged between diploid (top) and triploid (bottom) sibling axolotls. The half limbs were grafted to create a chimaeric (half triploid/half diploid) but otherwise normal limb which was amputated immediately.

histological sections of these limbs showed a normal skeletal pattern in 12 of the 13 cases. The remaining limb possessed four digits and appeared normal from gross observation but was found to be lacking the skeletal components of digit **4**.

Table 1 shows the observed frequencies of trinucleolate cells in the dorsal and ventral dermal preparations of the control and experimental limbs. We have used these data to further quantitate the extent of cellular contribution from graft and host tissues and the extent to which cellular displacement occurs during regeneration. Table 2 gives the real triploid frequencies (observed frequency/control frequency) in the regenerated limbs. Also included are the data used to determine the relative contributions of graft and host tissues to the regenerate. The extent of cellular contribution from the triploid half limb was determined separately for dorsal and ventral dermis by summing the real triploid frequencies for all the digits

Table 1. *Observed frequencies of trinucleolate cells in dorsal and ventral dermal preparations*

			Digit				
Limb	Graft	d/v	1	2	3	4	Control
Anterior grafts							
20-lt	3N	d	0·64	0·43	0·04	0·06	0·76
		v	0·55	0·22	0·09	0·06	
26-rt	3N	d	0·48	0·52	0·09	0·07	0·73
		v	0·67	0·24	0·06	0·03	
28-rt	3N	d	0·48	0·54	0·25	0·10	0·64
		v	0·25	0·21	0·25	0·36	
21-lt	2N	d	0·08	0·37	0·66	0·59	0·76
		v	0·21	0·65	0·70	0·72	
27-rt	2N	d	0·13	0·60	0·69	0·65	0·73
		v	0·18	0·66	0·73	0·73	
31-rt	2N	d	0·07	0·14	0·63	0·64	0·73
		v	0·14	0·18	0·55	0·70	
Posterior grafts							
20-rt	3N	d	0·21	0·40	0·28	0·49	0·76
		v	0·44	0·49	0·55	0·58	
26-lt	3N	d	0·03	0·05	0·42	0·62	0·73
		v	0·09	0·10	0·65	0·70	
28-lt	3N	d	0·14	0·29	0·49	0·56	0·64
		v	0·10	0·15	0·50	0·59	
30-lt	3N	d	0·05	0·17	0·58	0·51	0·73
		v	0·04	0·09	0·47	0·66	
27-lt	2N	d	0·61	0·59	0·43	0·12	0·73
		v	0·67	0·52	0·12	0·10	
29-lt	2N	d	0·53	0·46	0·23	0·07	0·64
		v	0·52	0·39	0·16	0·09	
31-lt	2N	d	0·56	0·62	0·42	0·17	0·73
		v	0·63	0·70	0·13	0·10	

The data in this table represent the observed trinucleolate frequencies in dorsal and ventral dermal preparations of each experimental digit, and the observed trinucleolate frequency in control triploid digits as a whole.

of a limb, then dividing by the number of digits scored. This analysis gave triploid contribution values (T.C.V. in Table 2) for the dorsal and ventral sides of the limb.

Table 2. *Real triploid frequencies (observed frequency/control frequency) in regenerated limbs*

			Real triploid frequency Digit					Total CV	
Limb	Graft	d/v	1	2	3	4	TCV	A	P
Anterior grafts									
20-lt	3N	d	0·84	0·57	0·05	0·08	0·38	0·34	0·66
		v	0·72	0·29	0·12	0·08	0·30		
26-rt	3N	d	0·66	0·71	0·12	0·10	0·39	0·37	0·63
		v	0·92	0·33	0·08	0·04	0·34		
28-rt	3N	d	0·75	0·84	0·39	0·16	0·53	0·48	0·52
		v	0·39	0·33	0·39	0·56	0·41		
21-lt	2N	d	0·11	0·49	0·87	0·78	0·56	0·34	0·66
		v	0·28	0·86	0·92	0·95	0·75		
27-rt	2N	d	0·18	0·82	0·95	0·89	0·71	0·25	0·75
		v	0·25	0·90	1·00	1·00	0·78		
31-rt	2N	d	0·10	0·19	0·86	0·88	0·50	0·48	0·52
		v	0·19	0·25	0·75	0·96	0·53		
							x̄ =	0·38	0·62
Posterior grafts									
20-rt	3N	d	0·28	0·53	0·37	0·64	0·45	0·43	0·57
		v	0·58	0·64	0·72	0·76	0·67		
26-lt	3N	d	0·04	0·07	0·58	0·85	0·38	0·54	0·46
		v	0·12	0·14	0·89	0·96	0·52		
28-lt	3N	d	0·22	0·45	0·77	0·88	0·58	0·45	0·55
		v	0·16	0·23	0·78	0·92	0·52		
30-lt	3N	d	0·07	0·23	0·79	0·70	0·44	0·56	0·44
		v	0·05	0·12	0·64	0·90	0·42		
27-lt	2N	d	0·84	0·81	0·59	0·16	0·60	0·54	0·46
		v	0·92	0·71	0·16	0·14	0·48		
29-lt	2N	d	0·83	0·72	0·36	0·11	0·50	0·48	0·52
		v	0·81	0·61	0·25	0·14	0·45		
31-lt	2N	d	0·77	0·85	0·58	0·23	0·60	0·57	0·43
		v	0·86	0·96	0·18	0·14	0·53		
							x̄ =	0·51	0·49
							Total x̄ =	0·45	0·55

The real triploid frequency data in this table were derived from the data in Table 1 as follows: the observed trinucleolate frequency for each dorsal and ventral dermal preparation of each digit was adjusted by dividing it by the relevant trinucleolate control frequency, to give a real triploid frequency. The triploid contribution value (T.C.V.) for each dorsal and ventral dermal preparation was derived by dividing the sum of the real triploid frequencies for all four digits by the number of digits (4). Anterior and posterior contribution values (total C.V.) were derived as follows: the mean T.C.V. for each limb was calculated from the T.C.V.s of each dorsal and ventral preparation. This value is then the fraction of the cells in the regenerate contributed by the triploid half. The contribution from the diploid half is 1·00 minus the contribution from the triploid half. Values underlined are those in which contribution from both anterior and posterior is close to 50 % (between 40 % and 60 %).

In theory, the resulting triploid contribution values could range from 0·0, where no triploid cells contribute, to 1·00, where only triploid cells contribute to the regenerate. A triploid contribution value of 0·5 indicates that cells from the triploid side contribute one-half of the total number of cells in the preparation, the other half being derived from diploid cells. The actual data show both a range of values from 0·30 to 0·78, as well as variability in triploid contribution values between the dorsal and ventral regions of the regenerate. The anterior and posterior contribution values (total CV in Table 2) were determined by first calculating the contribution from the triploid half, then subtracting that value from 1·00 to calculate the contribution from the diploid half. For example, limb 28-Rt regenerated from an anterior half triploid and posterior half diploid stump. The triploid contribution values were 0·53 and 0·41 for dorsal and ventral dermal preparations respectively. The triploid contribution value for the limb as a whole was calculated as the average of the dorsal and ventral triploid contribution values, or 0·48. This value represents the anterior (triploid) contribution to the regenerated limb (48 %). The remaining 52 % of the cells were contributed from the posterior (diploid) half of the original limb.

Overall, the diploid and triploid contribution values (total C.V.) for all limbs demonstrate two important points. First, the majority of grafted half limbs contributed approximately the same numbers of cells to the regenerated limb as did the ungrafted half limb. This is illustrated by the fact that 9 of the 13 limbs (69 %) had contribution values which fell between 0·4 and 0·6 (underlined in Table 2). Second, the remaining limbs (4/13 – 31 %) which had contribution values of less than 0·4 and greater than 0·6 were all cases in which the anterior half limb had been grafted, and all showed a reduced contribution from the anterior grafted tissue. Overall, the mean contribution value from limbs in which the anterior half was grafted was 0·38, suggesting that grafts of anterior tissue in general contribute fewer cells to the regenerated limb than do grafts of posterior tissue (mean contribution value = 0·49). However, it is important to note that we observed two cases in which limbs with anterior grafts had contribution values very close to 0·5, thus indicating that anterior grafted tissue is actually capable of contributing half of the total number of cells of the regenerate.

The triploid frequency data in Table 2 were used in quantitate the cellular displacement which occurred during limb regeneration (Tables 3A and 3B). The extent of cellular displacement was calculated for each dermal preparation (dorsal and ventral) by normalizing the triploid frequency (Table 2) to that fraction of all triploid cells present in an individual digit. To do this, the triploid frequency for each digit (dorsal or ventral half) was divided by the sum of the frequencies of all four digits in a given dermal preparation. This figure then represents the fraction of all triploid cells present in that particular half digit, and overall gives data on where triploid cells are found relative to one another in the limb as a whole. However, this analysis of cellular distribution in one direction (triploid into diploid) would be valid only if overall cellular contribution from diploid and triploid tissues were equal, but we have already shown that there is some

variability in overall cellular contribution to the regenerate from graft and host tissues. Consequently, we also performed the reciprocal analysis to determine the distribution of diploid cells across the whole limb. The diploid frequency for each dorsal and ventral half digit was determined as 1·00 minus the triploid frequency shown in Table 2. Thus, we have analysed bidirectional cellular distribution from triploid tissue into diploid tissue and *vice versa*. Tables 3A and 3B give the results of this analysis divided into cases in which the posterior half was triploid (Table 3A) or the anterior half was triploid (Table 3B). The separate data for each dorsal and ventral dermal preparation have been averaged and this mean value ($\bar{x}$) represents the relative distribution of cells in that digit. The overall mean frequencies of cellular distribution in each digit of diploid and triploid tissues are given at the bottom of each column and the arrows indicate the direction of cellular displacement which must have occurred to account for this distribution. For example, limb 28-lt, which possessed a grafted triploid posterior half and a host diploid anterior half, was found to have 15 % of all triploid cells in digit **2** and 9 % in digit **1**. Therefore, 24 % of all triploid cells were displaced from their side of origin (posterior) to the opposite side of the limb (anterior), the majority being displaced into the digit adjacent to the graft (digit **2**). Similarly, 13 % of all diploid cells were present in digit **3** while 5 % were present in digit **4**, thus showing that 18 % of diploid cells had been displaced from their side of origin (anterior) to the opposite side of the limb (posterior), the majority again being displaced into the immediately adjacent digit **3**.

The data generated from this analysis (summarized in Table 4) demonstrate that overall, approximately 24 % of the cells in the regenerated limb were observed on the opposite side of the limb from that on which they had originated. This finding is the same regardless of whether the displacement was from diploid limb regions into triploid limb regions or *vice versa*. However, there was a slight difference in the final distribution of cells from posterior *versus* anterior half limbs: 27 % of posterior cells were displaced while 21 % of anterior cells were displaced. This difference between anterior and posterior is correlated with the reduced contribution value for anterior tissue, thus suggesting that the amount of displacement which occurs is governed by the relative quantity of contributing cells. The distribution of the displaced cells was not random; most of the displaced cells (63 %) were observed in the digit adjacent to the graft/host interface, and the remaining displaced cells (37 %) were observed in the most peripheral digit.

DISCUSSION

A detailed quantitative analysis of the cellular contribution to regenerated limbs from anterior and posterior halves of the lower arm of axolotls is presented. The limb stumps from which these regenerated limbs arose had been grafted such that the anterior and posterior half limbs differed in ploidy. The data show that the regenerated limb comprises approximately half triploid and half diploid cells. The distribution of the cells indicates that about 25 % of the cells originating in one half

of the stump have become displaced to the opposite side of the regenerated limb. The bulk of the displaced cells are found in the region which is directly adjacent to their side of origin.

Although numerous tissue grafting experiments have been performed on the regenerating urodele limb, only a few such studies have made use of markers to trace cell lineage. Still fewer studies have been performed in a way which permits a detailed quantitative analysis of the data. One reason for this is that it has only recently become apparent that the triploid/diploid cell marker system can be useful in quantitative analyses provided that control cell counts for each donor animal are obtained, and that artifacts due to sectioning are bypassed wherever possible by the use of whole-mount dermal preparations (Muneoka *et al.* 1984). Furthermore, we have shown that contribution analyses based on dermal preparations correlate with those based on counts in cartilage (Muneoka & Bryant, 1984*a*), thus making the use of dermal preparations a relatively convenient means to estimate cellular contribution to the regenerate as a whole. The cellular contribution data we have presented show that during normal regeneration from the lower limb both anterior and posterior halves of the limb contribute approximately equal numbers of cells to the final regenerate. It appears, however,

Table 3A. *Cellular displacement during limb regeneration with posterior half triploid*

		Digit								
		1		2			3		4	
Limb	d/v	3N	2N	3N	2N		3N	2N	3N	2N
Posterior 3N										
21-lt	d	0·05	0·51	0·22	0·29		0·39	0·07	0·35	0·13
	v	0·09	0·73	0·29	0·14		0·31	0·08	0·32	0·05
	x̄	0·07	0·62	0·25	0·21		0·35	0·07	0·33	0·09
27-rt	d	0·06	0·71	0·29	0·16		0·33	0·04	0·31	0·09
	v	0·08	0·88	0·29	0·12		0·32	0·00	0·32	0·00
	x̄	0·07	0·79	0·29	0·14		0·33	0·02	0·31	0·05
31-rt	d	0·05	0·46	0·09	0·41		0·42	0·07	0·43	0·06
	v	0·09	0·44	0·12	0·41		0·35	0·14	0·45	0·02
	x̄	0·07	0·45	0·11	0·41		0·39	0·11	0·44	0·04
20-rt	d	0·15	0·33	0·29	0·22		0·20	0·29	0·35	0·17
	v	0·21	0·32	0·24	0·28		0·27	0·22	0·28	0·18
	x̄	0·18	0·33	0·27	0·25		0·23	0·25	0·31	0·17
26-lt	d	0·03	0·39	0·05	0·38		0·38	0·17	0·55	0·06
	v	0·06	0·47	0·07	0·46		0·42	0·06	0·45	0·02
	x̄	0·05	0·43	0·06	0·42		0·40	0·11	0·50	0·04
28-lt	d	0·09	0·46	0·19	0·33		0·33	0·14	0·38	0·07
	v	0·08	0·44	0·11	0·40		0·37	0·12	0·44	0·04
	x̄	0·09	0·45	0·15	0·37		0·35	0·13	0·41	0·05
30-lt	d	0·04	0·42	0·13	0·35		0·44	0·10	0·39	0·14
	v	0·03	0·41	0·07	0·38		0·37	0·16	0·53	0·04
	x̄	0·03	0·41	0·10	0·37		0·41	0·13	0·46	0·09
3N	x̄ =	0·08		0·18		←	0·35		0·40	
2N	x̄ =		0·50		0·31	→		0·12		0·08

that the anterior and posterior halves of the limb are differentially sensitive to the grafting procedure. In most cases (4/6), when the grafted tissue is anterior, the resulting regenerated limb consists of slightly more cells of posterior than of anterior origin. When anterior tissue is the host half of the limb, and even in some cases (2/6) where the anterior half is the grafted side, the anterior half contributes approximately 50 % of the cells. We interpret these findings to suggest that under ideal conditions each limb half contributes about half of the total number of cells of the regenerated limb, but that anterior tissue is more sensitive to graft-induced damage. This point is of importance with regard to the interpretation of tissue grafting experiments in general. Most such experiments are performed without cellular markers and are analysed at the level of the final morphology of the

Table 3B. *Cellular displacement during limb regeneration with anterior half triploid*

		Digit							
		1		2		3		4	
Limb	d/v	3N	2N	3N	2N	3N	2N	3N	2N
Anterior 3N									
27-lt	d	0·35	0·10	0·34	0·12	0·25	0·26	0·07	0·53
	v	0·48	0·04	0·37	0·14	0·08	0·41	0·07	0·42
	$\bar{x}$	0·41	0·07	0·35	0·13	0·17	0·33	0·07	0·47
29-lt	d	0·41	0·09	0·36	0·14	0·18	0·32	0·05	0·45
	v	0·45	0·09	0·34	0·18	0·14	0·34	0·08	0·39
	$\bar{x}$	0·43	0·09	0·35	0·16	0·16	0·33	0·07	0·42
31-lt	d	0·32	0·15	0·35	0·10	0·24	0·27	0·09	0·49
	v	0·40	0·08	0·45	0·02	0·08	0·44	0·07	0·46
	$\bar{x}$	0·36	0·11	0·40	0·06	0·16	0·35	0·08	0·47
20-lt	d	0·55	0·07	0·37	0·17	0·03	0·39	0·05	0·37
	v	0·60	0·10	0·24	0·25	0·10	0·32	0·07	0·33
	$\bar{x}$	0·57	0·09	0·31	0·21	0·07	0·35	0·06	0·35
26-rt	d	0·42	0·14	0·45	0·12	0·08	0·37	0·06	0·37
	v	0·67	0·03	0·24	0·25	0·06	0·35	0·03	0·37
	$\bar{x}$	0·55	0·09	0·29	0·19	0·07	0·36	0·05	0·37
28-rt	d	0·35	0·13	0·39	0·09	0·18	0·33	0·07	0·45
	v	0·23	0·26	0·20	0·29	0·23	0·26	0·34	0·19
	$\bar{x}$	0·29	0·19	0·29	0·19	0·21	0·29	0·21	0·32
3N	$\bar{x}$ =	0·44		0·34		➤ 0·14		0·09	
2N	$\bar{x}$ =		0·11		0·16 ◄		0·34		0·40

The distribution data in this table were derived from those in Table 2 as follows: 3N values: the real triploid frequency for each dorsal and ventral half digit was divided by the sum of the frequencies of the four digits in each dermal preparation. The mean triploid distribution value for each digit (average of dorsal and ventral) is shown in the table ($\bar{x}$) and represents the fraction of all triploid cells present in a particular digit. 2N values: The distribution of diploid cells was determined by first calculating the real diploid frequency as 1·00 minus the real triploid frequency for individual dorsal or ventral sides of each digit. The remainder of the calculation was the same as that described above for 3N values. The values at the bottom of the columns represent the overall mean distribution of 2N and 3N cells and the arrows indicate the direction of cellular displacement.

Table 4. *Summary of cellular displacements*

Type	Final distribution of cells	Original half limb	Adjacent digit	Peripheral digit
Posterior	3N	0·75	0·18	0·08
	2N	0·74	0·16	0·11
		$\bar{x} = 0·74$	0·17	0·10
Anterior	3N	0·79	0·14	0·09
	2N	0·81	0·12	0·08
		$\bar{x} = 0·80$	0·13	0·08
	Overall	$\bar{x} = 0·77$	0·15	0·09

The data in this table were derived from the mean distribution data in Table 3A,B. For the original half limb column, the fractions of cells in the two digits on the side of origin of the graft were summed.

regenerated limb. The assumption in such experiments is that grafted tissues survive and participate in regeneration in a manner analogous to that of ungrafted host tissues. Our data argue that this assumption cannot be made and point to the importance of utilizing cellular markers in experiments of this type.

The findings of this study are in agreement with studies in which the cellular contribution to the formation of supernumerary limbs has been analysed (Muneoka & Bryant, 1984*a*,*b*). In these studies supernumerary limbs resulting from contralateral blastema or limb bud grafts were found to comprise about equal numbers of cells of both anterior and posterior origin. Striking similarities such as this between normal limb regeneration and supernumerary limb formation argues for the existence of similar mechanisms to control limb outgrowth in each case.

One difference between the results reported here and those reported by Muneoka & Bryant (1984*a*,*b*) on supernumerary limbs concerns the extent of cellular mixing observed during limb outgrowth. During supernumerary limb formation, the boundary between diploid and triploid tissue is quite sharp, with little or no cellular mixing, whereas during normal limb regeneration 25 % of the cells are displaced away from their side of origin. This difference can be attributed to differences in the initial conditions prior to limb outgrowth. Regeneration from an amputated limb stump is preceded by wound healing of the amputation surface. During wound closure and the initiation of regeneration, it has been proposed that cells from different positions in the limb circumference become redistributed in the plane of amputation, bringing positionally different cells into proximity, and thus creating the conditions under which the intercalary growth required for normal limb regeneration can take place (Bryant *et al.* 1981). Such an initial redistribution of cells can account for the final cellular displacement observed in the present study. Conversely, the development of supernumerary limbs is not preceded by the closure of a major wound, since such limbs arise as a result of bringing cells from dissimilar positions into close proximity by grafting. Hence,

extensive mixing of cells away from their side of origin would not be expected and has been shown not to occur in the formation of supernumerary limbs.

Our data concerning the extent of displacement of cells show both similarities to and differences from the previously reported data of Tank *et al.* (1985) and Rollman-Dinsmore & Bryant (1984). Tank *et al.* (1985) analysed the final distribution of marked cells in limbs regenerated from half diploid/half triploid upper arms, and Rollman-Dinsmore & Bryant (1984) looked at the final distribution in regenerates of cells derived from small grafts of triploid skin in diploid upper limbs. In both studies the extent of cellular displacement was assessed by determining what fraction of all observed trinucleolate cells were found in different regions of the regenerate. This type of analysis gives information about the distribution of one of the two populations of cells, but provides no information about the other. In our analysis the relative cellular distribution was derived from normalized real frequency data, and hence information is available concerning the distribution of diploid as well as triploid cells. Rollman-Dinsmore & Bryant (1984) found that from small triploid skin grafts trinucleolate cells were observed throughout the regenerated limb, showing that the progeny of dermal cells can become displaced considerable distances during regeneration. Our finding that marked cells are found in all regenerated digits is consistent with these previous data, although the results cannot be directly compared because Rollman-Dinsmore & Bryant (1984) did not collect real frequency data. Our data are also consistent with those of Tank *et al.* (1985) who showed that from half triploid/half diploid upper arms about 25 % of all trinucleolate cells were displaced to the opposite side of the regenerated limb. However, Tank *et al.* (1985) found no marked cells displaced to the digit furthest from the grafted half limb, whereas we find that 37 % of all displaced cells were observed in the most peripheral digit.

Overall, the picture of limb regeneration which emerges from these analyses is one in which cells at the plane of amputation are involved in a roughly proportional way in the regeneration of the limb. They argue against any strictly mosaic relationship between cells in one part of the circumference at the limb base and cells in the same part of the circumference at more distal levels within the regenerated limb, since a quarter of all the cells involved in the outgrowth are displaced to the opposite half of the limb. The displacement, which is bidirectional, that is, from graft side to host side and *vice versa*, is consistent with the idea that limb regeneration is generated by intercalation as a product of interactions between cells from different regions in the limb.

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REFERENCES

BRYANT, S. V., FRENCH, V. & BRYANT, P. J. (1981). Distal regeneration and symmetry. *Science* **212**, 993–1002.

FRENCH, V., BRYANT, P. J. & BRYANT, S. V. (1976). Pattern regulation in epimorphic fields. *Science* **193**, 969–981.

MUNEOKA, K. & BRYANT, S. V. (1984*a*). Cellular contribution to supernumerary limbs in the axolotl, *Ambystoma mexicanum. Devl Biol.* **105**, 166–178.

MUNEOKA, K. & BRYANT, S. V. (1984*b*). Cellular contribution to supernumerary limbs resulting from the interaction between developing and regenerating tissues in the axolotl. *Devl Biol.* **105**, 179–187.

MUNEOKA, K., WISE, L. D., FOX, W. F. & BRYANT, S. V. (1984). Improved techniques for use of the triploid cell marker in the axolotl, *Ambystoma mexicanum. Devl Biol.* **105**, 240–245.

ROLLMAN-DINSMORE, C. & BRYANT, S. V. (1984). The distribution of marked dermal cells from small localized implants in limb regenerates. *Devl Biol.* **106**, 275–281.

TANK, P. W., CARLSON, B. M. & CONNELLY, T. G. (1976). A staging system for forelimb regeneration in the axolotl, *Ambystoma mexicanum. J. Morph.* **150**, 117–128.

TANK, P. W., CONNELLY, T. G. & BOOKSTEIN, F. L. (1985). Cellular behavior in the anteroposterior axis of the regenerating forelimb of the axolotl, *Ambystoma mexicanum. Devl Biol.* **109**, 215–223.

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