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Journal
Developmental Biology, 105(1)

ISSN
0012-1606

Authors
Muneoka, Ken
Wise, L David
Fox, Warren F
et al.

Publication Date
1984-09-01

DOI
10.1016/0012-1606(84)90280-x

DEVELOPMENTAL BIOLOGY 105, 240-245 (1984)

Improved Techniques for Use of the Triploid Cell Marker in the Axolotl, *Ambystoma mexicanum*

KEN MUNEOKA, L. DAVID WISE,[1] WARREN F. FOX, AND SUSAN V. BRYANT

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

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

Techniques for using the triploid cell marker for studying cell lineage during the development and regeneration of the axolotl limb are described. Triploid animals possess cells with three nucleoli while diploid animals possess cells with two nucleoli. We have developed a technique for isolating the limb dermis as a sheet of cells for whole-mount analysis of cellular ploidy. Whole-mount tissue preparations as well as paraffin-embedded sectioned tissues were stained specifically for nucleoli with bismuth. Cell counts from a number of triploid and diploid dermal preparations show that (1) diploid dermal cells never possess three nucleoli, (2) the frequency of trinucleolate cells in whole-mount triploid dermal preparations is not 100% but varies between animals from 30 to 76%, (3) within a single triploid animal, the frequency of trinucleolate cells in different dermal preparations is constant. These data establish the usefulness of this technique and emphasize the need for appropriate control cell counts when using the triploid cell marker in the axolotl.

INTRODUCTION

The development and characterization of cellular markers for experimental use have been instrumental in furthering our understanding of the basic cellular processes guiding embryonic development. For example, the chick/quail cellular marker system (Le Douarin, 1969) has been used extensively in a number of different experimental systems to ask questions about the fate of grafted cells during the development of the chick embryo. Recently, Tompkins and co-workers (1984) have developed a tetraploid strain of *Xenopus laevis* as a marker for studies of cell lineage during the development of the vertebrate eye. In studies using urodeles, the triploid cell marker in the axolotl, *Ambystoma mexicanum*, is the only endogenous cell marker currently available for experimental use (Steen, 1968; Namenwirth, 1974; Dunis and Namenwirth, 1977; Slack, 1980, 1983; Pescitelli and Stocum 1980; Thoms and Fallon, 1980). We have been using this marker for cell lineage studies on developing and regenerating axolotl limbs (Muneoka and Bryant, 1984a,b).

The triploid cell marker has several characteristics which are desirable in a useful marker. First, triploid animals can be produced easily and in large numbers by exposing fertilized eggs to high hydrostatic pressure (Gillespie and Armstrong, 1979). Second, the cell marker is autonomous. Triploid cells are distinguishable from diploid cells by the number of nucleoli per cell; triploid cells possess three nucleoli while diploid cells possess two (Fankhauser and Humphrey, 1943). Third, triploid animals develop normally, and the behavior of triploid cells appears to be identical to that of diploid cells. Thus, this marker is attractive for experimentally probing the extent of cellular involvement in developing and regenerating tissues of the axolotl. Unfortunately, use of the triploid cellular marker has been rather limited due to difficulties associated with the accurate identification of the number of nucleoli per cell. These difficulties have centered not only on the problems of the reliability and specificity of the nucleolar staining, but also on the inherent problem of counting nucleoli in sectioned material. We have overcome both of these problems by modifying a nucleolus-specific bismuth staining procedure (Locke and Huie, 1977) so that it is effective on axolotl tissue (Muneoka *et al.*, 1983) and by developing techniques for whole-mount analysis of bismuth-stained tissue, thus eliminating any artifacts due to sectioning.

In analyzing whole-mount preparations of the limb dermis from a number of triploid animals, we found that there is a great deal of variability in the frequency of trinucleolate cells from one animal to another. This is an important observation since previous investigators have tended to assume that the frequency of trinucleolate cells is constant from animal to animal and have consequently attributed any variation in this frequency to sectioning artifacts (Steen, 1968; Namenwirth, 1974). Despite variation from animal to animal, we are encouraged by our finding that there is very little variability in the trinucleolate cell frequency of different preparations from within a single animal. The cell

[1] Present address: Warner-Lambert Company, Pharmaceutical Research Division, 2800 Plymouth Road, Ann Arbor, Mich. 48105.

0012-1606/84 $3.00

marker can therefore become very useful provided that appropriate control cell counts are made for each donor triploid animal. This paper focuses specifically on the various techniques involved in the use of the triploid cell marker in the axolotl.

MATERIALS AND METHODS

The animal used in this study was the axolotl, *A. mexicanum*, spawned at the University of California, Irvine. Triploid animals were made by subjecting fertilized eggs, collected at 20-min intervals, to 8 min of hydrostatic pressure (6000 psi) 1 hr after egg collection (Gillespie and Armstrong, 1979). Treated animals were screened for triploidy in two separate ways. Initially, squashes of tail tip epidermis were analyzed by phase microscopy for cells with three nucleoli. However, we found that this screening procedure sometimes gave inaccurate results, so we adopted the procedure of routinely screening bismuth-stained whole-mount preparations of skinned digital cartilages of young larvae following the staining protocol described below.

Sectioned tissue. Mature limbs, regenerating blastemas, and developing limb buds were amputated and fixed in Carnoy's fixative for 1–2 days, then stored in 70% ethanol. Mature and regenerated limbs were decalcified in 10% Versene, (ethylene dinitrilotetraacetic acid) at pH 6.0–6.5 for up to 3 days depending on the size and age of the tissue. Decalcified limbs were rinsed well in distilled water, dehydrated, cleared, embedded in paraffin, sectioned at 10 μm and stained with bismuth (see below).

Dermal preparations. We have developed a technique whereby the dermis of fixed, mature axolotl limbs can be prepared as a sheet of cells for whole-mount analysis (Fig. 1). Limbs were fixed in Carnoy's fixative and transferred to 100% ethanol. The dorsal and ventral skin (epidermis and dermis) was peeled from the underlying tissues of the limb after cutting along the anterior and posterior margins of the limb and trimming the skin from the interdigital webbing (Fig. 1a). The isolated sheets of dorsal and ventral skin were transferred to distilled water and pinned out on wax with tungsten tacks (Fig. 1b). In distilled water the epidermis can be carefully separated from the dermis with fine watchmaker forceps and a small blunt glass probe. Epidermis was left at the tips of the digits and at the proximal edge of the skin to prevent the dermis from curling up. The isolated dermis (Fig. 1c) was carefully floated onto a gelatin-coated slide and covered with a siliconized coverslip. The dermis was compressed onto the side by drawing water from beneath the coverslip with filter paper. The slide was quickly frozen on dry ice and the coverslip was removed. The slide with the dermis attached was either stored in 70% ethanol or processed immediately using the bismuth-staining protocol described below. To control for contamination of the dermal preparations by epidermis, the isolated dermis from 10 limbs was embedded in paraffin, serially sectioned at 10 μm, stained with Mallory's triple stain, and analyzed for the presence of epidermal cells.

Staining. This staining procedure is a modification of Locke and Huie's (1977) bismuth-staining procedure and

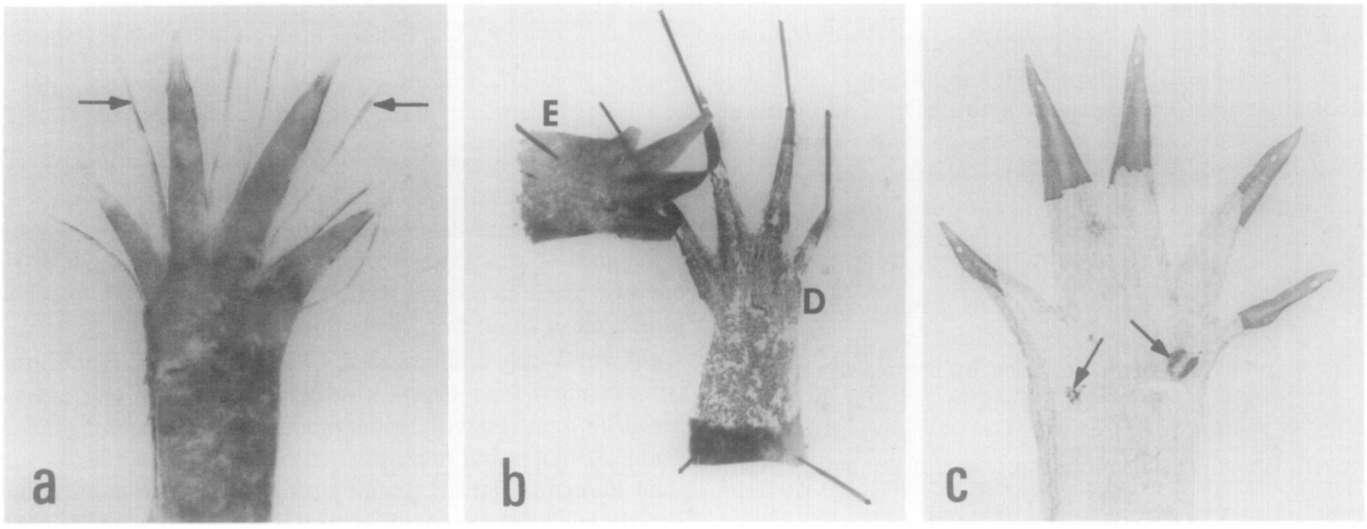

FIG. 1. Procedure for isolating the dermis of an axolotl limb. The limb is fixed in Carnoy's fixative for 1–2 days and transferred to 100% ethanol. (a) The skin is cut along the anterior and posterior margin of the limb and the interdigital skin (arrows) is trimmed away. The dorsal and ventral skin is peeled off and transferred to distilled water. (b) The dorsal skin is shown pinned out on wax and the epidermis (E) has been carefully peeled away from the dermis (D). Epidermis is left at the tips of the digits and at the proximal edge of the skin to prevent the dermis from curling up. (c) The dermis is mounted on a slide and stained with bismuth. In this example the ventral dermis of a hindlimb is shown. Arrows indicate parts of contaminating epidermis obvious in whole-mount preparations. ×7.

can be used for sectioned material as well as for whole-mount tissue preparations.

1. Deparaffinize and/or hydrate tissue	
2. Postfix in 10% buffered formalin phosphate, pH 7.0	1 hr
3. Rinse in distilled water	5 min
4. *Optional step* for depigmentation of dermal preparations:	
a. Treat with 0.25% potassium permanganate	3–5 min
b. Rinse in distilled water	5 min
c. Treat with 1.0% oxalic acid	1 min
d. Rinse in distilled water	1 min
5. Rinse in 0.1 *M* triethanolamine pH 7.0	30 min
6. Stain in bismuth-staining solution	overnight
7. Rinse in 0.1 *M* triethanolamine pH 7.0 (2×)	10 min
8. Treat with ammonium sulfide (1:300 in 0.1 *M* triethanolamine)	10 min
9. Rinse in distilled water	
10. Dehydrate, clear, and coverslip.	

The stock bismuth of Locke and Huie (1977) was made as follows: 20 g of sodium tartrate (Fisher) was dissolved in 500 ml of 1 *N* sodium hydroxide. This solution was added slowly to 10 g of bismuth subnitrate (Mallinckrodt, Paris, Ky.) and magnetically stirred until the bismuth was in solution. The staining solution was made by diluting the stock solution 1:3 with 0.2 *M* triethanolamine–HCl buffer and adjusting the final pH to 7.0 (Locke and Huie, 1977). Although the diluted staining solution is stable for several days, we routinely make it up fresh for more uniform results.

Cell counts. We have performed counts of trinucleolate cells in sectioned cartilage and muscle and in whole-mount dermal preparations using bright field illumination at 600×. In sectioned tissue, the frequency of trinucleolate cells was calculated as 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 or cells which had an ambiguous nucleolar count were not scored. The frequency of trinucleolate cells in dermal preparations was determined as the number of cells with three nucleoli divided by the total number of scorable cells. Cells were determined to be unscorable if no nucleolus was evident, or if nuclei overlapped, thereby obscuring the exact number of nucleoli per cell.

RESULTS AND DISCUSSION

The modification of Locke and Huie's bismuth staining procedure described here and in Muneoka *et al.* (1983), was found to provide good overall staining of nucleoli in paraffin sections of developing limb buds (Fig. 2a), regenerating blastemas (Fig. 2b), and differentiated tissues (Figs. 2c, d) and in whole-mount preparations of the dermis (Figs. 3a, b), cartilage (Fig. 3c), and epidermis (Fig. 3d) of the axolotl limb. The nucleoli stained dark brown against a light brown granular nucleus. The cytoplasm stained faintly or not at all in epidermal, dermal, cartilage, blastemal, and limb bud cells. Myofibrils of muscle tissue stained light brown as did the extracellular connective tissue matrix of the dermis. This light background staining was found to be useful for the identification of tissue types, yet at the same time it allowed the intensely stained nucleoli to stand out clearly.

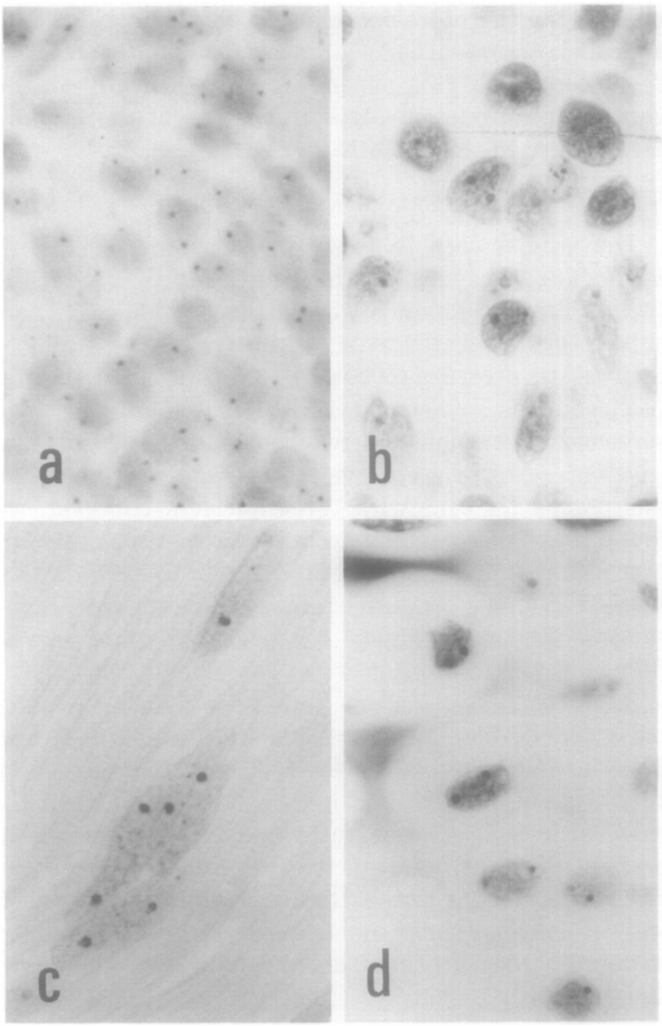

FIG. 2. Bismuth-stained paraffin sections of limb tissues. Nucleoli stain intensely against the more lightly staining nucleus. (a) Limb bud mesenchyme. ×575. (b) Blastema mesenchyme. ×575. (c) Skeletal muscle. ×680. (d) Cartilage. ×680.

The development of the technique for making whole-mount preparations of dermis has greatly facilitated the analysis of cell lineage during supernumerary limb formation in the axolotl (Muneoka and Bryant, 1984a,b).

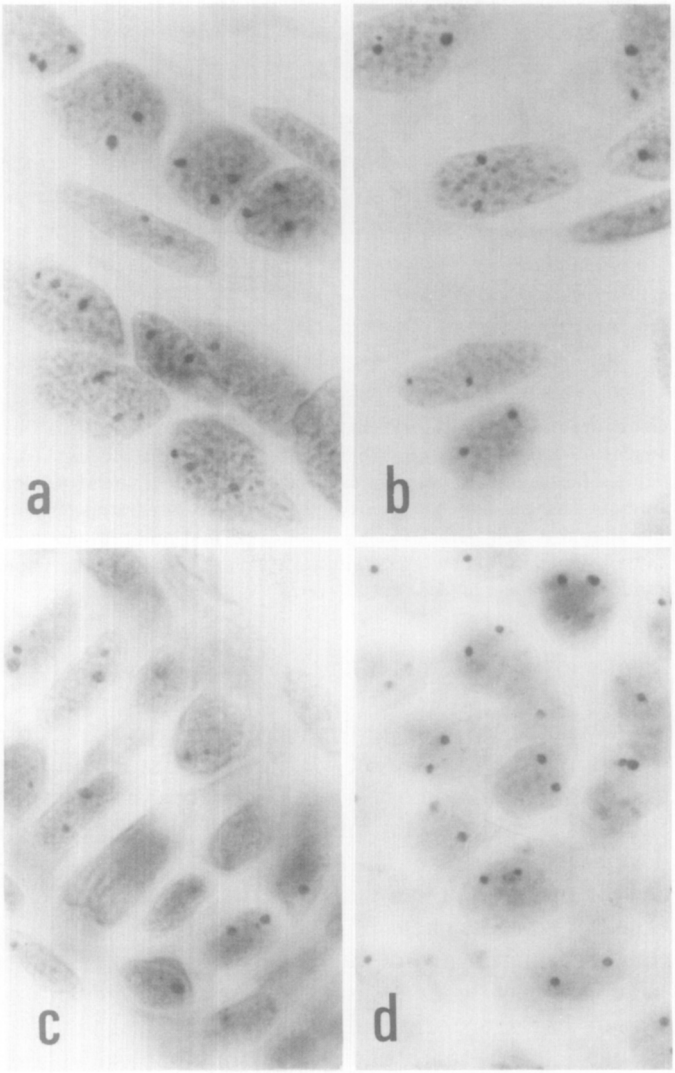

FIG. 3. Bismuth-stained whole-mount preparations of limb tissues. (a) Triploid dermis. (b) Diploid dermis. (c) Cartilage. Whole skinned digits were stained with bismuth and prepared for whole-mount analysis. This example shows cartilage at the tip of a digit. (d) Epidermis left at the digit tips of a dermal preparation. ×680.

An example of a bismuth stained dermal preparation is shown at low magnification in Fig. 1c. Figures 3a and b show higher magnifications of bismuth-stained triploid and diploid dermal cells in whole-mount dermal preparations. Dermal cells in whole-mount preparations stained with bismuth are easily scored for ploidy provided there is no overlap of nuclei. Although contaminating epidermal cells are quite distinct in these whole-mount preparations (e.g., see Fig. 1c), we also checked for their presence in serial sections of 10 dermal preparations. We found no contaminating cells on the epidermal surface of the dermis (Fig. 4). The analysis of serially sectioned dermal preparations also showed that only the outermost layer of dermal cells is present in these preparations.

A number of factors were found to influence the ease of making and analyzing the dermal preparations. (1) The age of the limb. Regenerating limbs 4- to 8-weeks old proved to be ideal material from which to prepare and analyze whole mounts of the dermis. With younger regenerates it was extremely difficult to separate the epidermis from the dermis without destroying the dermis, while in older regenerates the complexity of the dermis, particularly the degree of nuclear overlap, created problems for analysis. (2) The length of time of tissue storage in 70% ethanol. We found that the longer tissue was stored in 70% ethanol, the harder it was to separate the epidermis from the dermis. We have made whole-mount dermal preparations of material which had been stored for 2 years in 70% ethanol, but it was extremely tedious and the success rate was low. We now routinely make dermal preparations of limbs which have not been stored after fixation. (3) Depigmentation of the dermis. Following the potassium permanganate/oxalic acid depigmentation procedure, bismuth-stained cells were scorable but the background nuclear staining was clearly increased and the definition of the boundary of individual dermal cell nuclei was reduced. Whenever possible, we now avoid using the depigmentation procedures.

To establish a baseline for analysis of triploid frequencies, it was necessary to make counts on diploid tissue (in paraffin sections and in whole-mount dermal preparations) to establish the frequency (if any) of known diploid cells that appeared triploid. These data appear in Table 1. Dermal preparations from 10 diploid limbs were analyzed, and no cells appearing to possess

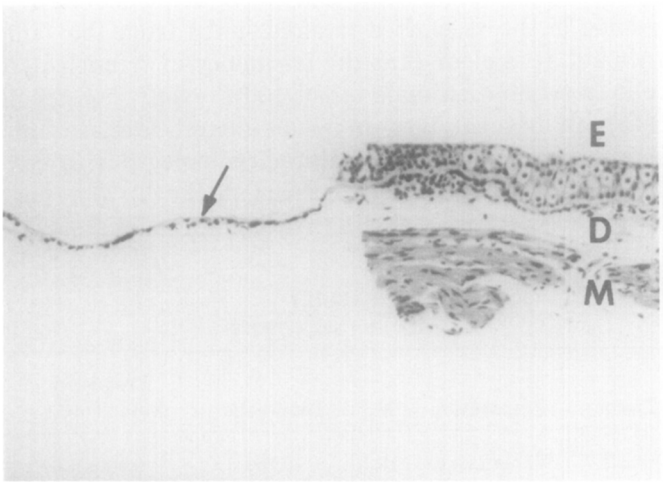

FIG. 4. Paraffin section of a dermal preparation. Full thickness skin (epidermis, E; dermis, D; and muscle, M) is shown on the right. The dermis was isolated from the underlying muscle and the overlying epidermis as described in the text. Only the more peripheral dermal cells are present in the final dermal preparation (arrow). No contamination with epidermal cells was observed. ×40.

three nucleoli were observed (total number of cells scored = 10,769). Serial longitudinal sections of cartilage and muscle from 7 diploid limbs were similarly analyzed. The frequency of diploid cartilage cells which appeared to possess three nucleoli was very low (0.003; total cells counted = 7415) while in muscle tissue, the frequency of apparent triploid cells was much higher (0.024; total cells counted = 7236). The apparently trinucleolate cells in sectioned tissue are thought to be the result of two nuclei overlapping each other in such a way that they appear to be a single nucleus. The difference in frequency of apparent triploid cells in diploid muscle and diploid cartilage is probably a reflection of both the difference in nuclear shape and in cell density of the two cell types. Nuclei of cartilage cells tend to be spherical and are not densely distributed in the tissue, whereas the nuclei of muscle cells are elongated and have a closely packed distribution in the tissue. Also the plane of section is an important factor to be considered when scoring for triploid cells in muscle tissue (Steen, 1968; Namenwirth, 1974). Transverse sections of muscle tissue cannot be scored because such a small portion of the nucleus is revealed. Longitudinal sections are probably ideal for analysis, but in between these two extremes there is a great deal of ambiguity. Furthermore, the degree of contraction of the muscle affects the amount of nuclear overlap and thus increases the difficulty of attaining accurate nucleolar counts.

We have performed cell counts on dermal preparations from a number ($N = 18$) of triploid animals to compare the frequency of trinucleolate cells from one animal to another (Fig. 5). We have also made an analysis of the trinucleolate frequency in triploid limbs which were grafted onto diploid hosts and of the trinucleolate frequency in the ungrafted triploid limbs from the same animals. It is clear that the frequency of trinucleolate cells in triploid animals is not 100%, even when whole cells can be examined in whole-mount preparations. Furthermore, the trinucleolate frequency varies considerably from one animal to the next (Fig. 5). The range of trinucleolate frequencies in dermal preparations was

TABLE 1
DIPLOID CELL COUNTS

Tissue	Preparation	N	Total cells	Frequency of trinucleolate (%)
Dermis	Whole mount	10	10,769 ($\bar{x}$ = 1077/limb)	0.000 (0%)
Cartilage	Section	7	7415 ($\bar{x}$ = 1059/limb)	0.003 (0.3%)
Muscle	Section	7	7236 ($\bar{x}$ = 1034/limb)	0.024 (2.4%)

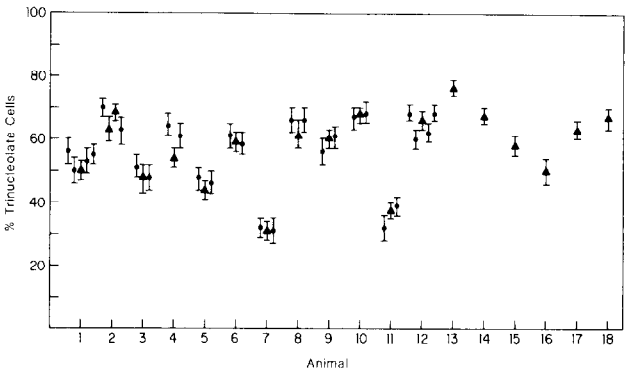

FIG. 5. Comparison of the percentage of trinucleolate cells in whole-mount dermal preparations from 18 triploid animals. Cell counts from ungrafted triploid limbs (▲) are compared to similar cell counts from triploid limbs grafted onto diploid hosts (●) for 12 of the 18 triploid animals. There is much variability in the trinucleolate frequency from one animal to another, but cell counts of dermal preparations from any single animal show great similarity. Bars represent the upper and lower 95% confidence limits.

from a low of 0.30 to a high of 0.76. On the other hand, there was very little variation in the trinucleolate frequency between different dermal preparations from the same animal regardless of whether the tissue had been grafted to a diploid host or not. Therefore the variation between animals is not an artifact of the protocol for dermal isolation or of the staining procedure, but a reflection of animal to animal variations in the frequency of cells with three nucleoli. From this finding we conclude that meaningful use of the triploid cell marker must include control counts from within the same animal rather than from triploid animals in general.

Slack (1980) recently showed that immunological graft rejection resulting from skin grafts between triploid and diploid axolotls is delayed sufficiently that it does not affect the formation of supernumerary limb structures, but that it does interfere with the scorability of the triploid cell marker. Similarly, we find that in whole-mount preparations, a dermis which is undergoing immunological rejection is filled with lymphocytes (of host origin when scorable) making the analysis of these preparations impossible. When rejection of skin grafts does occur, the rejection is most severe in the stump region of the limb and may often be absent in the regenerated limb, despite the presence of cells derived from the skin graft (Wise and Muneoka personal observation). We attribute this observation to the fact that cells participating in limb regeneration are going through a number of changes (dedifferentiation, active proliferation, redifferentiation) which may affect their antigenicity, while the grafted skin cells in the stump region remain virtually unchanged and are therefore probably very susceptible to immunological rejection. This conclusion is further supported by the observation

that tissue rejection following limb bud or blastema grafting experiments is rare (Muneoka, personal observation). Similar observations have been previously reported by de Both (1970).

In conclusion, the techniques we have described here make triploidy a useful cell marker for studies of cell lineage in the axolotl. The major drawback of this cell marker lies in the fact that not all cells of a triploid animal appear triploid (by nucleolar counts) thus necessitating stringent control frequency analyses. However, once this limitation is taken into account, the triploid cell marker can become a powerful tool in the investigation of cell behavior during both development and regeneration.

We thank Peter Bryant, David Gardiner, and Christine Rollman-Dinsmore, for critically evaluating the manuscript and Gregory Holler for technical assistance in the preparation of the manuscript. This work was supported by grants from the National Institutes of Health, HD 07029 and HD 06082.

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