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*Short Contribution*

# Mononuclear leukocytes in the newt limb blastema: *in vitro* behavior

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**ABSTRACT Since the precise interactions of the various newt limb blastema cell types (pleiomorphic, bipolar, signet and multinucleated cells) with one another are difficult to ascertain *in vivo*, in this study we describe the *in vitro* behavior and interactions of these cell types with one another. The data show that the mononuclear signet cells interact (fuse) with other signet cells or fuse directly with the multinucleated cells. Further, they indicate that the signet cells are in fact mononuclear leukocytes and the giant cells are osteoclasts. Therefore, we conclude that these two cell types are not formed by dedifferentiation but are of hematopoietic origin and may play minor roles in blastema production.**

KEY WORDS: *limb regeneration, blastema cells, culture, osteoclasts*

In the field of amphibian limb regeneration there are still many pieces of the puzzle which are poorly understood. One area deals with the production of progenitor (blastema) cells which form beneath the wound epithelium and further differentiate into more specialized structures necessary for the complete regenerate. The regenerative ability which urodeles, such as the newt *Notophthalmus viridescens*, possess is a direct result of the formation of these dedifferentiated blastema cells. The amputation of a limb results in the initial formation of a wound epidermis beneath which a pool of dedifferentiated cells accumulates. These blastema cells will proliferate until cues are given to induce the re-differentiation of these cells into new cell types (Tsonis, 1991). These cells contain the positional information necessary for a complete, normal regenerate (Stocum, 1984). Many researchers have studied these blastema cells *in vitro* to determine their cellular nature and characteristics. Their results have yielded a quartet of cells which make up the regeneration blastema. These cell types are: 1) pleiomorphic (Fimian, 1959; Jabaily *et al.*, 1982; Ferretti and Brockes, 1988); (2) bipolar (Ferretti and Brockes, 1988); 3) large multinucleate (Fimian, 1959); and 4) signet (Maier and Miller, 1992). The precise nature and interaction of the aforementioned cell types with each other remains to be further elucidated.

The role of osteoclasts in the process of regeneration was proposed many years ago through the studies of Fimian (1959) and Hay and Fischman (1961). When the limb of a newt, for example, is amputated through any of the ossified bones, there is an induction of the formation of osteoclasts to aid in the repair/reproduction of the damaged bone. Osteoclasts are large multinucleated cells which are a type of macrophage derived from the hematopoietic tissue in the bone marrow (Hancox, 1949; Nijweide and de Grooth, 1992). Studies *in vivo* using tritiated thymidine incorporation have demonstrated that during the newt limb regeneration process the mononuclear leukocytes are produced from hematopoietic origin (Hay and Fischman, 1961; Fischman and Hay, 1962). Subsequent fusion of these cells results in the formation of multinucleate "giant cells" (osteoclasts). These results (Hay and Fischman, 1961; Fischman and Hay, 1962) clearly demonstrate that the formation of osteoclasts in the regenerating newt limb *in vivo* occurs by cells produced from hematopoietic origin and not by the dedifferentiation of stump tissues. We believe that the fourth blastema cell type identified as the signet cell by Maier and Miller (1992) is in fact one of these mononuclear leukocytes. Its role in the regeneration blastema remains unclear. Here in this study the *in vitro* behavior of these "signet cells" with other cell types which are comprised in the blastema population is examined.

The cells which migrate from the explanted blastema tissue comprise a population of different cells (Fig. 1A). As can be seen in Table 1, the pleiomorphic cells make up the majority of the cell population. The less abundant cell types, the osteoclasts (giant, multinucleate cells which can have at least 2 nuclei or as many as 100) and the bipolar make up 1-2% of the total population each. The bipolar cells have been postulated to be nerve cell remnants or possibly differentiated myocytes. The mononuclear leukocytes — a grouping which includes monocytes and lymphocytes — seem to be abundant (up to 45% of the total) and with an increase in this cell type, the incidence of cell-cell interactions (fusions) increases. These percentages are in accordance with other studies (Fimian, 1959; Ferretti and Brockes, 1988; Maier and Miller, 1992). These mononuclear cells have a very distinct appearance. They have a very high nucleus-to-cytoplasm ratio and also contain numerous granules (Fig. 1B). All of these blastema cell types express the regeneration-specific antigen, 22/18. This antigen was proposed to be expressed in regeneration-associated tissues (Ferretti and

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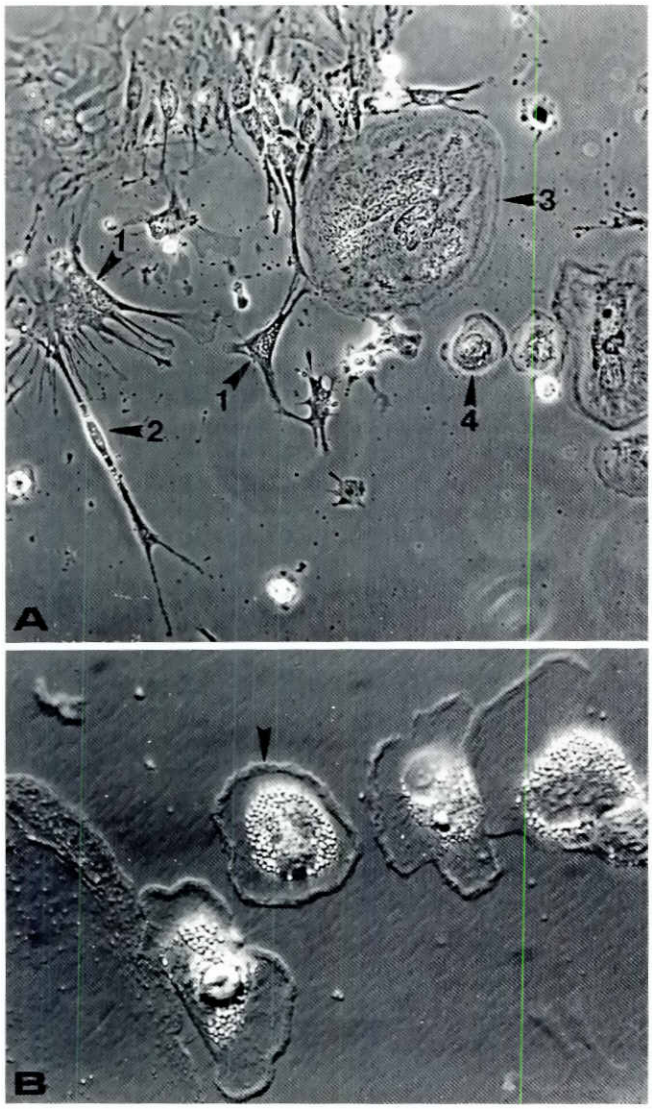


**Fig. 1. Blastema cell types. (A)** *In vitro blastema cell types showing representatives from the 4 cell populations. 1) pleiomorphic cells; 2) bipolar cells; 3) osteoclasts; 4) signet cells (mononuclear leukocytes). x10.* **(B)** *Morphology of the mononuclear cells in vitro observed under Hoffman phase optics. Individual cells displaying the high nucleus cytoplasm ratio, a ruffled border (arrow) and presence of granules. x40.*

Brockes, 1988). The mononuclear leukocytes and the osteoclasts express the 22/18 antigen as seen in Fig. 2B. This reactivity may be induced by cell division. Both of these criteria were used by Maier and Miller (1992) in the classification of these cells as a new cell type.

In order to determine whether these signet cells were actually mononuclear leukocytes, we established criteria to ensure that the cells we identified as signet were the same to those observed by Maier and Miller (1992) and then proved conclusively that these cells are mononuclear leukocytes. The first criterion was the morphology of the signet cells. Autofluorescence after exposure to direct UV light was the second criterion. The third criterion was the interactions (fusion) of the mononuclear leukocytes with each other as well as direct fusion with pre-existing giant cells. The fourth criterion was the *in vivo* labeling of mononuclear leukocytes with tritiated thymidine (Hay and Fischman, 1961). These criteria provide the necessary clues for affirming that the signet cells are in fact mononuclear leukocytes which arise from hematopoietic origin and not from dedifferentiation of stump tissues. The cells observed in the *in vitro* cultures have a distinct phenotype as already shown (Fig. 1B). Morphologically we have identified the same cells as Maier and Miller (1992). This phenotype is also similar to that of various white blood cells. When exposed to direct UV light, these cells also autofluoresce (data not shown). The giant cells also autofluoresce (as expected since these cells contain granules similar to those observed within the mononuclear cells). Therefore, we conclude (by fulfilling 2 criteria) that these cells are in fact the same cell type observed by Maier and Miller (1992) and that morphologically these cells are similar to white blood cells.

The third criterion involves the interactions among the mononuclear cells and with the giant cells. The mononuclear cells

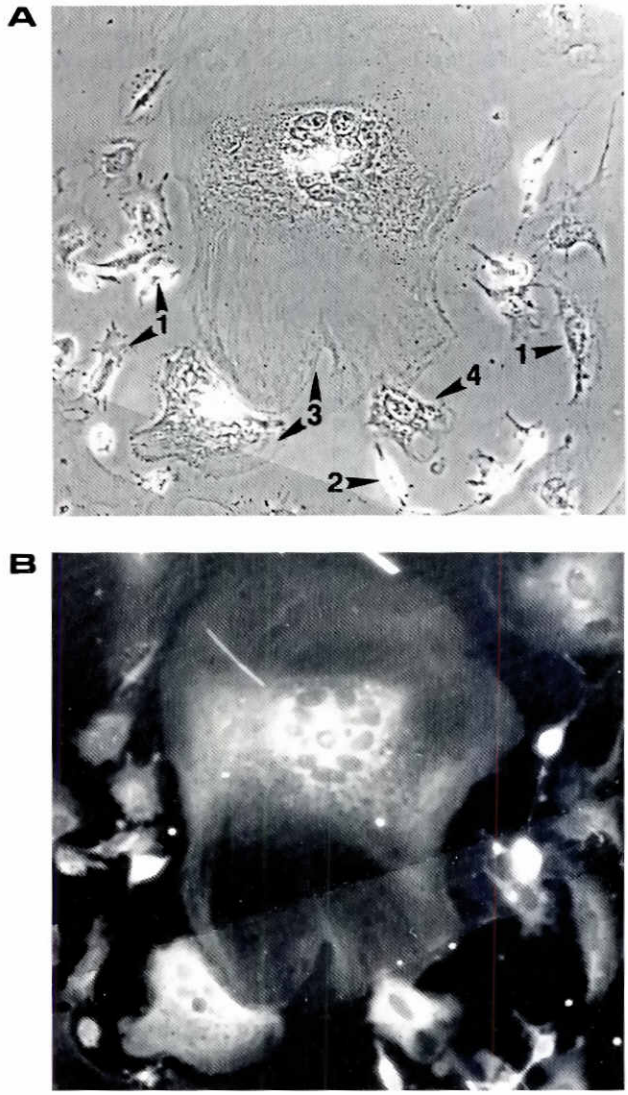


**Fig. 2. 22/18 staining of the blastema cells *in vitro*. (A)** *Phase contrast image of the various cell types.* **(B)** *Corresponding fluorescent image depicting 22/18 expression in all 4 cell types from the blastema. Numerical labels: 1) pleiomorphic cells; 2) bipolar cells; 3) osteoclasts; 4) mononuclear leukocytes. There was no nuclear staining observed since the 22/18 antigen is cytoplasmic. x25*

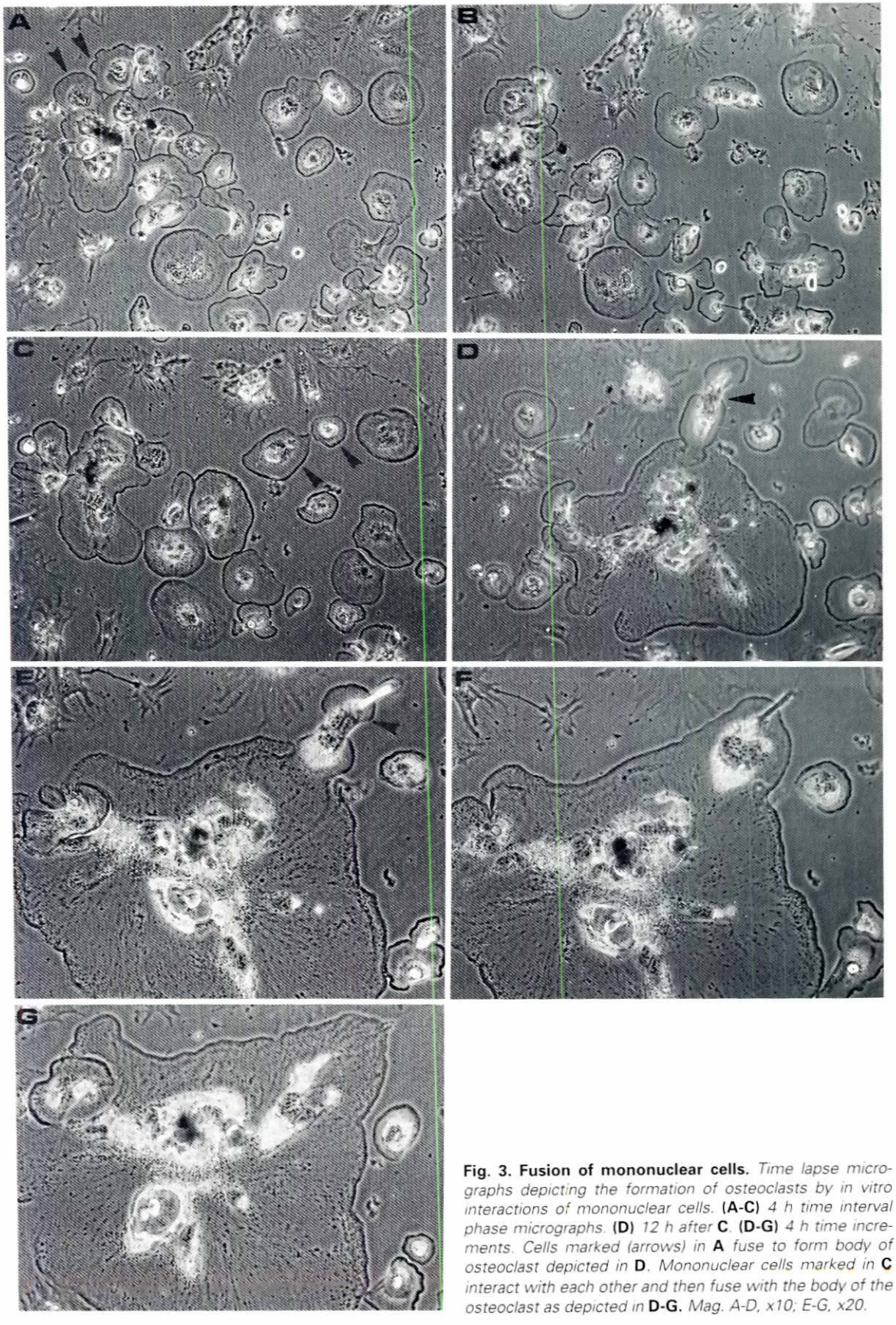


**Fig. 3. Fusion of mononuclear cells.** *Time lapse micrographs depicting the formation of osteoclasts by in vitro interactions of mononuclear cells.* **(A-C)** *4 h time interval phase micrographs.* **(D)** *12 h after* **C**. **(D-G)** *4 h time increments. Cells marked (arrows) in* **A** *fuse to form body of osteoclast depicted in* **D**. *Mononuclear cells marked in* **C** *interact with each other and then fuse with the body of the osteoclast as depicted in* **D-G.** *Mag. A-D, x10; E-G, x20.*

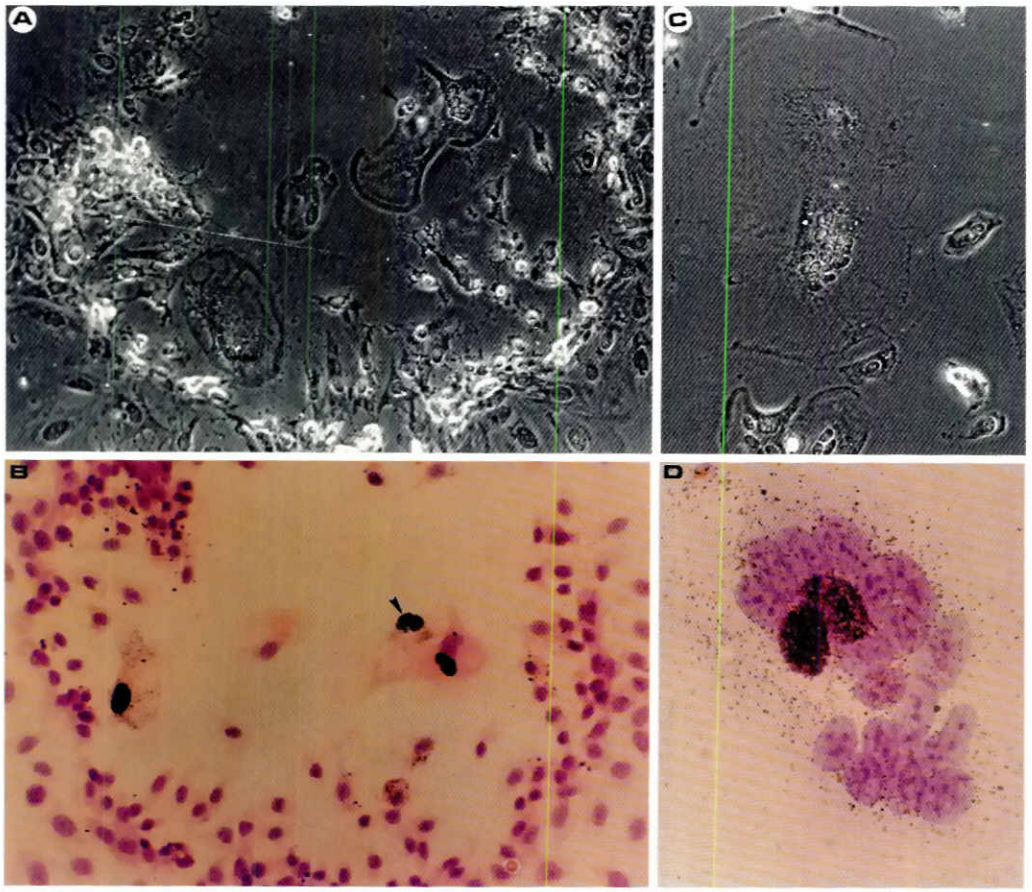


**Fig. 4. Single nuclei labelled *in vivo* fused with osteoclasts.** *In vitro culture of blastema from tritiated thymidine-injected animals. The animals were injected prior to amputation and blastema was explanted 2 weeks later.* **(A)** *Phase contrast image of cells prior to autoradiography showing presence of various cell types.* **(B)** *Same cells as in* **A** *after autoradiography processing and counterstaining with hematoxylin and eosin. Note that only the nuclei from the mononuclear leukocytes and osteoclasts are positive. Two nuclei from a cell fusing with an osteoclast are marked (arrow) in* **A** *and* **B**. **(C)** *Phase contrast image of an osteoclast containing many nuclei (20) and* **(D)** *autoradiography processed osteoclast showing that only 2 of the 20 nuclei are positive. Distorted cell morphology in* **A** *and* **B** *(as compared to* **C** *and* **D***) was due to fixation and position of the micrographs. Mag. A-C, x10; D, x40.*

will fuse with one another (depicted in Fig. 3) thus becoming binucleate, then in turn fusing with yet another giant cell. These interactions have been shown to be the process by which osteoclasts are formed (Hancox, 1949; Fischman and Hay, 1962; Nijweide and de Grooth, 1992). Such interactions are shown in Fig. 3 where time lapse photography depicts the cells fusing *in vitro*. The cells were first observed as single (Fig. 3A, top left of micrograph marked with arrows), but as time proceeded, they fused to form the body of the osteoclast in Fig. 3D. Also, in Fig. 3C two cells begin to interact with each other and then subsequently fuse with the same osteoclast (sequentially in Fig. 3C-G). These data indicate that these cells are in fact mononuclear leukocytes fusing to form osteoclasts. We never observed the pleiomorphic or bipolar cells interacting (fusing) with either the mononuclear leukocytes or osteoclasts.

The final criterion involved the *in vivo* labeling of mononuclear leukocytes with tritiated thymidine. In the case of the newt limb regeneration system, such labeling prior to amputation results in the labeling of epidermal cells and circulating white blood cells (Hay and Fischman, 1961; Fischman and Hay, 1962). As seen in Fig. 4A-B, of the cells which migrated out of the blastema explant, only 2 cell types displayed nuclear thymidine labeling, the mononuclear leukocytes and the osteoclasts. There were no pleiomorphic cells displaying positive nuclei. Therefore, these mononuclear signet cells are leukocytes and the giant cells are osteoclasts.

The overall results of this study indicate that of the 4 blastema cell types, possibly only 2 may arise from the process of dedifferentiation while the other 2 are from hematopoietic stem cells. This was determined by the *in vitro* interactions from which the osteoclasts were formed. Our data suggest that the pleiomorphic cell population comprises almost 90% of the population at a given time and therefore are probably the only regeneration-specific cells.

## Experimental Procedures

### *Animals*

All newts, *Notophthalmus viridescens*, were obtained from Amphibians of North America, (Nashville, TN, USA), and kept at room temperature in aquaria. Animals were anesthetized in 3-aminobenzoic acid ethylester (Sigma) and the fore- and hindlimbs were amputated through the mid ulna-radius level. Two weeks post-amputation the wound epithelium was removed and the blastema was collected for *in vitro* culturing.

TABLE 1

**PERCENTAGES OF VARIOUS CELL TYPES FROM EXPLANTED NEWT BLASTEMAS**

| Cell Type | Percentage of blastema cell population | 22/18 Expression |
|---|---|---|
| Pleiomorphic | 60-90% | + |
| Bipolar | ≤1% | + |
| Osteoclasts (multinucleate cells) | 1-2% | + |
| Mononuclear Leukocytes | 30-45% | + |

***Cell culture***

Isolated blastemata were rinsed in 50% L-15 medium containing 2% penicillin/streptomycin, and 2% fungizone for 15 min. Then the blastemata were plated on gelatin coated dishes and incubated at 25°C, 1% $CO_2$, in the following medium: 30% L-15, 30% EMEM, 9% FCS, 1% Pen/strep, 1% fungizone, insulin (10 μg/ml), 10% conditioned media, and 1% kanamycin. The conditioned media was collected from other newt cultures and added as a supplement for potential factors which newt cells may secrete. This media was changed 3 times per week. Blastema cells were observed migrating from the explants after 2 days and all cell types were present. Cells were examined for varying periods of time during which time course pictures were taken to observe the cell-cell interactions. Cells were also exposed to direct UV light for detection of autofluorescence in the cell types.

***Immunostaining***

All cells were stained with the 22/18 monoclonal antibody (Kintner and Brockes, 1985) to determine the reactivity of the cells to this regeneration-associated antigen (Kintner and Brockes, 1984, 1985). Briefly, the explants were removed and the cells were rinsed twice in 1XPBS (15 min each), fixed with cold acid/alcohol (95% ethanol/5% glacial acetic acid) and then rinsed as previous with 1xPBS. The cells were then pre-incubated with 65% L-15:EMEM (1:1) plus 10% FCS at 37°C. The primary antibody (22/18; Developmental Studies Hybridoma Bank) was then added and incubation was continued at 37°C for 1 h. Cells were washed in 1XPBS (3 times, 15 min each) and then 22/18 expression was detected by incubation of FITC-conjugated secondary antibody (FITC-goat anti-MIgM; μ-chain-specific; Cappel) for 1 h at 37°C. The cells were then washed with 1xPBS and mounted in 90% glycerol, 10% 1xPBS, and 0.2M n-propyl gallate (Sigma; to reduce photo-bleaching) and observed under UV microscopy.

***Labeling of mononuclear leukocytes with tritiated thymidine***

Newts were injected with tritiated thymidine (5 μCi intraperitoneally; Amersham, 81 Ci/mmol) 1 day prior to amputation of the forelimbs at the mid ulna-radius level. After 14 days of regeneration, the blastemata were aseptically removed and cultured *in vitro* on gelatin coated coverslips. Explants were monitored for migration of the various cell types from the explants. When observations were complete, the cells were fixed in methanol:acetic acid (3:1) for 20 min and air dried. Then the coverslips were attached to slides dipped in Kodak NTB-2 emulsion diluted (1:1) and exposed for 3 weeks. The slides were developed in Kodak D-19 developer (2.5 min, 15°C), fixed (Kodak fixer, 7 min; 25°C) and counterstained with hematoxylin and eosin for observation under bright field or dark field microscopy.

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