DETERMINATION OF THE BLASTEMA CELL CYCLE IN REGENERATING LIMBS OF THE LARVAL AXOLOTL, *AMBYSTOMA MEXICANUM*[1]

WILLIE D. McCULLOUGH AND ROY A. TASSAVA, Department of Zoology and Developmental Biology Program, The Ohio State University, Columbus, Ohio 43210

Abstract. Total cell cycle time as well as time of S phase, $G_2+\frac{1}{2}$ M phase, and $G_1+\frac{1}{2}$ M phase was determined for blastema cells of 9 day nerve-dependent forelimb regenerates of larval axolotls. Nine days post-amputation the limbs had early cone blastemas which continued regeneration (on those larvae not fixed for histology) and by day 23 had 4 digit regenerates. The mean mitotic index for 9 day blastemas was 2.65% with 79.5% of the mitotic figures labeled 8 hrs post ^{3}H-thymidine injection and 96% at 16 hours. Mean cycle time, as determined from the time from the 1st 50% intercept to the 3rd 50% intercept, was 40 hrs. The S phase, $G_2+\frac{1}{2}$ M, and $G_1+\frac{1}{2}$ M were 32 hrs, 5.6 hrs, and 2.4 hrs respectively.

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In the unamputated salamander limb essentially no cells of the mesodermal tissues are synthesizing DNA or undergoing mitosis (Kelly and Tassava, 1973; Hay and Fischman, 1961). However, within a few days after amputation, dedifferentiation and entry of the mesodermal cells into the cell cycle occur with subsequent DNA replication and mitosis. The progeny cells accumulate to form the blastema from which the new limb develops (Hay and Fischman, 1961; Thornton, 1968; and Tassava and Mescher, 1975).

Recent reports have suggested that injury, nerves, and the wound epidermis are essential to regeneration because of influences on the different phases of the cycle of the limb cells (Tassava and Mescher, 1975; Mescher and Tassava, 1975). To test this hypothesis, it will be important to measure accurately the different phases of the cycle of limb stump cells and blastema cells, particularly under experimental conditions. The literature documents a single study (Grillo, 1971) on cell cycle analysis for blastema cells which utilized the adult newt, *Notophthalmus viridescens*. The aims of the present study were to extend blastema cell cycle analysis to the larval axolotl, *Ambystoma mexicanum*, and to compare these findings to cell cycle parameters reported for the adult newt by Grillo (1971).

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MATERIALS AND METHODS

Larvae of the Mexican axolotl, *Ambystoma mexicanum*, kindly provided by Dr. R. R. Humphrey, Indiana University, were grown to an average weight of 1.58±0.14 gm and snout-tail tip length of 6.11±0.27 cm. Larvae were maintained in individual containers at 21.5± 1°C in conditioned water.

Forelimbs were amputated through the distal third of the humerus and, after 9 days of regeneration, at which time early cone blastemas were present, each larva was given 1.5 μCi of H^3-thymidine in 0.05 ml sterile H_2O (H^3-methyl thymidine, Sp. Act. 71.6 Ci/mM, New England Nuclear) by i.p. injection through the tail musculature (Kelly and Tassava, 1973). Left forelimbs of all larvae were denervated on day 8. Amputations, denervations, and injections were done while larvae were anesthetized in MS 222 (Kelly and Tassava, 1973).

At two hrs post-injection, limbs of three larvae were fixed in Carnoy's fixative (1:3, glacial acetic acid:absolute alcohol). Thereafter, limbs of 3 larvae were fixed at each 8 hr interval post-injection, through 80 hrs. Limbs were histologically prepared, serially sectioned longitudinally at 10 μ, and short ribbons, containing 5 sections each, were distributed on 4 slides such that each slide contained representative regions of the limb (Kelly and Tassava, 1973). A single slide of each limb was randomly chosen for Feulgen staining of mitotic figures and autoradiography.

Each blastema was sampled in the middle and

on the two sides for labelled mitotic figures using the humerus as a landmark. A total of 600–700 cells/blastema were sampled for mitotic index determinations. The mitotic index (M.I.) was calculated as follows: Number of mitotic figures/total no. of cells sampled x 100= M.I. Any mitotic figure which had 4 or more silver grains was considered labelled. A total of 50–60 mitotic figures/blastema were sampled for labelling. The means of 3 blastemas of each sample time were plotted through the experimental period (fig. 1).

Below we report the data from the regenerating right limbs only. According to the methods of Quastler and Sherman (1959) and Takahashi (1966), the following cell cycle parameters were determined from the plot of labelled mitotic figures of right forelimb blastemas (fig. 1): (1) cell cycle time (CCT) = time from first to third 50% intercept, (2) S phase = time from first to second 50% intercept, (3) $G_2+\frac{1}{2}$ M = time from 0 to first 50% intercept, and (4) $G_1+\frac{1}{2}$ M = CCT − ($G_2+\frac{1}{2}$ M+S).

RESULTS

At 9 days post-amputation, both left and right forelimbs had early cone blastemas. At 80 hrs post-injection, right limb blastemas had progressed to the paddle stage. Right limbs continued regeneration on those larvae which were not fixed for histology and by day 23 had 4 digit regenerates. In contrast, denervated left limb blastemas stopped development and were resorbed back to the amputation surface by 14 days post-denervation. The measurement of the cell cycle in these denervated blastemas

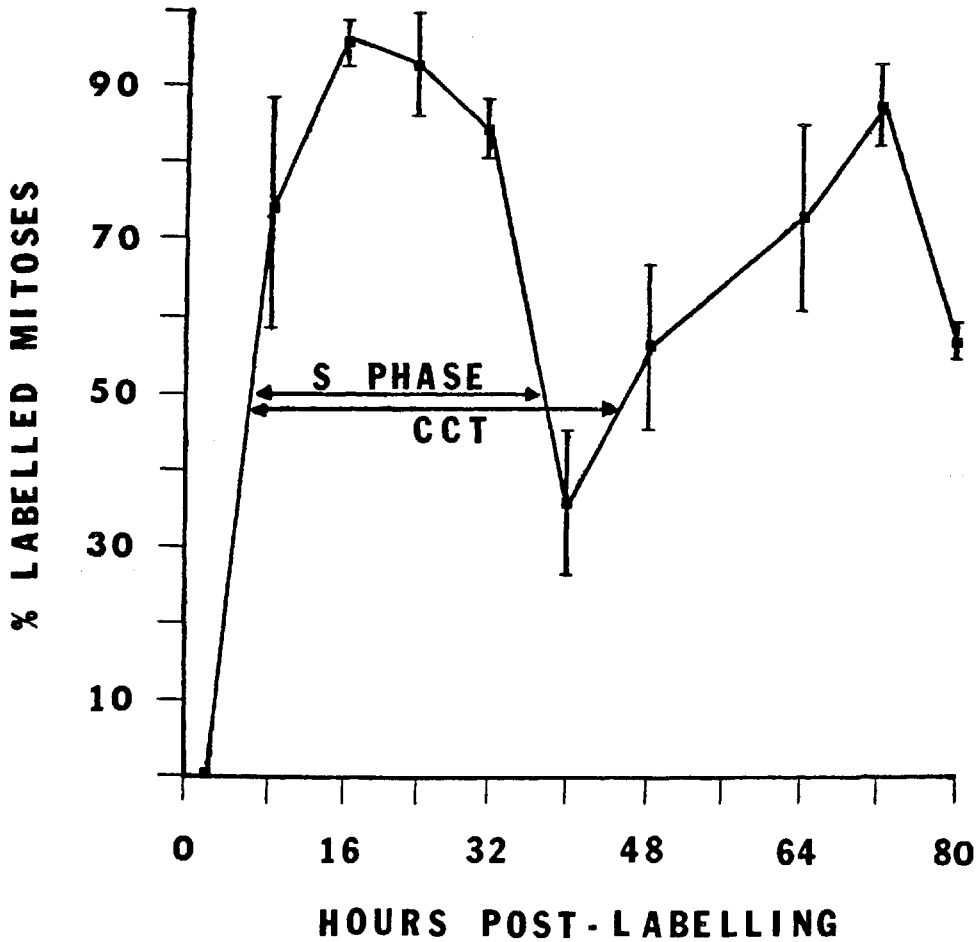

FIGURE 1. Percent labelled mitoses for 9 day axolotl blastemas sampled through 80 hrs post-labelling. Vertical lines represent the standard error of the mean for the three blastemas sampled at each point (S phase = DNA synthetic period; CCT = cell cycle time).

will be reported separately. The mean mitotic index of 9 day blastemas was found to be 2.65%. No labelled mitotic figures were observed for the first 2 hrs post-injection. However, 79.5% of the mitotic figures were labelled at 8 hrs post-injection, 96% at 16 hrs, 95% at 24 hrs, and 86% at 32 hrs. Labelled mitotic figures constituted only 34% of the total at 40 hrs post-injection followed by an increase to a second peak at 72 hrs (fig. 1). The mean cell cycle time (CCT) is determined as the time from the first 50% intercept to the 3rd 50% intercept, as shown in figure 1, and is 40 hrs in duration. Also, as can be seen in figure 1, $G_2+\frac{1}{2}$ M, $G_1+\frac{1}{2}$ M, and the S phase are 5.6 hrs, 2.4 hrs, and 32 hrs in duration respectively.

DISCUSSION

These results with larval axolotls are in close agreement with those obtained by Grillo (1971) for the adult newt. The newt 18 day blastema cell cycle time was reported to be 45 hrs and $G_2+\frac{1}{2}$ M, $G_1+\frac{1}{2}$ M, and the S phase were 4.5 hrs, 3.0 hrs, and 37.5 hrs respectively (Grillo, 1971). The 37.5 hr S phase of adult newt blastema cells represents 83% of the total cell cycle time. Larval axolotl blastema cells have an S phase of 32 hrs which is 80% of the total 40 hr cell cycle time. In both adult newts and larval axolotls, the G_1 phase of the cell cycle is the shortest portion. From the mitotic index determinations, the length of M can be calculated as follows: mitotic index of 9 day blastemas is 2.65%. Assuming a random cell population, this represents the percent of the total cell cycle time occupied by the M phase. Since the total cell cycle is 40 hrs, 2.65% of 40 hrs = 1.06 hrs. Thus, G_2 and G_1 can be calculated as well:

$G_2+\frac{1}{2}[1.06]=5.6$ hrs; $G_2=5.07$ hrs.

$G_1+\frac{1}{2}[1.06]=2.4$ hrs; $G_1=1.87$ hrs.

It is of interest that G_2 is considerably longer in duration than G_1. This may be significant in light of recent reports which indicate that nerves influence some G_2 event (Mescher and Tassava, 1975; Tassava *et al.*, 1974). It will now be possible to determine whether the G_1, G_2, or S phases of the 9 day blastema cell cycle are influenced by denervation, whether denervation affects any of these phases of older nerve-independent blastemas, and whether cells in different regions of a single blastema have different cycle times (Thornton, 1968).

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