Neurotrophic and X-ray blocks in
the blastemal cell cycle

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SUMMARY

Using microdensitometry techniques the points in the cycle where blastemal cells become blocked after X-irradiation or denervation of the regenerating amphibian limb have been identified. X-irradiation blocks the cells in both G, and G, and those cells that were in S at the time of irradiation presumably proceed to G,. After denervation, however, cells accumulate only in G, and those that were in S or G, continue through the cycle to the next G,.. The latter results are clearly contradictory to a recent theory proposing a G, neurotrophic control of blastemal cells and a solution to the contradiction is presented in the light of recent results.

INTRODUCTION

Regeneration of the amphibian limb is an epimorphic phenomenon and hence dependent upon a continued supply of new cells for restitution of the original pattern. Dedifferentiated cells of the stump provide this material by mitosis and a blastema appears at the tip of the limb. Thus if cell division is inhibited no regeneration will occur – a situation which prevails when the limb is either denervated or X-irradiated. Following denervation of the blastema, DNA synthesis declines (Singer & Caston, 1972) and the mitotic index falls to zero (Singer & Craven, 1948): a newt brain extract injected into the blastema reinitiates synthesis (Jabaily & Singer, 1977). Similarly after irradiation with a suitable dose of X-rays (> 1200R) a mitotic block is incurred (Maden & Wallace 1976; Wertz, Donaldson & Mason, 1976) and it is thus tempting to speculate that these two inhibitory treatments act in a similar fashion on some key point in the blastemal cell cycle. However, I report here that whereas irradiation blocks cells both in the G, or G, phases of the cell cycle, denervated blastemal cells accumulate only in G,.. This latter observation is clearly contrary to a recent theory proposing that nerves control the G, phase of the blastemal cell cycle (Tassava & Mescher, 1975).

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MATERIALS AND METHODS

The forelimbs of 12 axolotls, *Ambystoma mexicanum*, were amputated through the distal humerus and allowed to regenerate at 20 °C until cone stage blastemas had developed. Five animals then had their forelimbs denervated by crushing the nerves at the brachial plexus. The other five were irradiated with 2000R from a Newton and Victor Ltd X-ray machine at 90 kV, 5.5 mA. Two blastemas in each series were sampled at daily intervals for four days including one sample immediately after treatment (day 0). The remaining two animals were used as additional controls fixed on day 4. All blastemas were fixed in 3:1 alcohol:acetic acid, hydrolysed in 5 N-HCl for 1 h and stained in Feulgen for 2 h. They were tapped out in water after removing the epidermis and squashed under a coverslip. The absorption values of 100 single nuclei on each sample were read on a Vickers M85 scanning microdensitometer to determine the proportion of cells in each phase of the cell cycle. The cycle time of young axolotl blastemal cells is 2 days (Maden, 1976), thus by 4 days after treatment all the cells should have collected at any arrest point.

RESULTS

Figs. 1a and 2a are the histograms from control blastemas (day 0) showing typical distributions of DNA contents in rapidly dividing populations of cells (day 4 controls also showed these characteristics). It has previously been revealed (Maden, 1976) that in these blastemal cells the duration of S phase is about 60% of the total cycle time. We would therefore not expect any obvious bimodality in the Feulgen distributions of normal blastemas.

**Irradiation.** After irradiation (Figs. 1b and 1c) there was a gradual appearance of two distinct peaks as cells seemed to be lost from the middle (S phase). This suggests that cells which were in $G_1$ or $G_2$ at the time of irradiation are prevented from proceeding further through the cycle and cells undergoing DNA synthesis continue, perhaps to $G_2$, and then stop. However, this interpretation is probably too simplistic since in Fig. 1c the proportion of cells in $G_1$ (left-hand peak) is 65% and $G_2$ (right-hand peak) 35%. Had all those cells which were in S phase at the time of irradiation (60% of the population – see above) progressed to $G_2$ and then stopped there would clearly be far more cells in $G_2$ than $G_1$. Although the reason for the larger $G_1$ peak observed here remains to be elucidated, we can conclude that there seem to be two blocks after blastemal cells are irradiated.

**Denervation.** Following denervation, on the other hand, both the S phase and the $G_2$ cells gradually disappeared (Fig. 2b) leaving one massive $G_1$ peak (Fig. 2c). By 4 days after denervation almost all the cells were in $G_1$ and the height of this peak had increased threefold. Thus in contrast to the effects of irradiation described above, after denervation there seems only to be one block point and that is in $G_1$. 
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Fig. 1. Irradiation: Frequency of nuclei in classes of Feulgen absorption from (a) normal cone blastema, (b) blastema 2 days after irradiation with 2000R, (c) blastema 4 days after irradiation. (b) and (c) show that irradiated cells become blocked in $G_1$ and $G_2$.

Fig. 2. Denervation: Frequency of nuclei in classes of Feulgen absorption from (a) normal cone blastema, (b) blastema 2 days after denervation, (c) blastema 4 days after denervation. (b) and (c) show that cells collect in $G_1$ after denervation.

DISCUSSION

Radiobiological studies in other tissues (Davies & Evans, 1966) have identified both $G_1$ and $G_2$ blocks after irradiation and the above data show that axolotl blastemas are typical in this regard. These results complement the demonstration that after irradiation the amputated limb does not initiate DNA synthesis as normal (Wertz et al., 1976) and neither do mitoses appear (Maden & Wallace, 1976). The reason for this inhibition is therefore clear – the cells at the tip of the limb cannot leave the $G_1$ phase (or $G_0$) in which they have differentiated.

Concerning the effects of denervation, it has been concluded by Tassava and his colleagues (Tassava, Bennett & Zitnik, 1974; Mescher & Tassava, 1975;
Tassava & Mescher, 1975) that a neurotrophic factor is required for the passage of cells through the G₂ phase of the blastemal cell cycle. They arrived at this conclusion on the basis of the observation that when denervated limbs were amputated, the cells at the tip began to synthesise DNA but no mitoses appeared. However, this hypothesis has recently been challenged following the repetition and extension of this type of experiment (Maden, 1978). Not only were labelled cells and mitoses observed after denervated limbs were amputated, but denervation 6 days prior to amputation reduced the proportion of labelled cells and number of mitoses. It was therefore suggested that there is a pool of trophic factor in the limb. Now the results reported here show that denervated cells become blocked in G₁, so if the pool of trophic factor were to be totally removed from the limb before amputation there should be no cells entering S phase or mitosis. This experiment has yet to be performed. Thus it seems that this pool of trophic factor is responsible for the spurious observation of labelled cells in denervated limbs (Tassava et al. 1974) which led to the hypothesis of a G₂ trophic control.

Mescher & Tassava (1975) themselves report the results of microdensitometric analysis of sections of denervated limbs. They failed to show cells collecting in G₂ and suggested the reason for this failure was that G₂ blocked cells were selectively removed from the limb. This was not the case in G₂ blocked irradiated cells (Fig. 1c) which remained in the blastema, implying selective removal is not a real mechanism. However, microdensitometry of sections is notoriously inaccurate; indeed neither did Mescher & Tassava's data reveal a G₁ block. This problem was overcome here by the use of blastemal squashes to demonstrate that denervated cells accumulate in G₁.

Finally, the question of whether the trophic factor is a rate-limiting (by controlling the rate of G₁) or an all-or-nothing molecule (by controlling the number of cells cycling) has already been discussed (Maden, 1978). It is important to emphasize that the data reported here do not distinguish between these two possibilities as a greatly protracted G₁ phase would accumulate cells in G₁ just as a complete block. Other evidence from continuous labelling studies does, however, favour the rate-limiting hypothesis (Maden, unpublished).

In conclusion it is clear that although denervation and irradiation both inhibit cell division and hence limb regeneration, they seem to do so by different cellular mechanisms. The above results must surely lay to rest the hypothesis (Rose, 1962) that X-rays act by destroying the nervous component of the limb.

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REFERENCES


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