Irradiated cells and blastema formation in the adult newt, *Notophthalmus viridescens*

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**SUMMARY**

This investigation primarily consisted of labeling cells in forelimbs of the newt; irradiating the labeled limb; and then inducing regeneration with a non-irradiated skin graft. The labeled cells were obtained by injecting tritiated thymidine intraperitoneally into newts with regenerating right forelimbs. The cells in the regenerate which were undergoing DNA synthesis were labeled, and those which differentiated and ceased to divide remained labeled for long periods. The labeled regenerates were irradiated with 2000 R, and subsequently the irradiated limbs were re-amputated. Some limbs were supplied with a hind-limb skin autograft; others received no graft. The limbs without the grafts did not regenerate, whereas several of those with grafts did.

Histological studies of the blastemas of the new regenerates indicated that labeled, irradiated cells were released from the internal tissues near the amputation surface and were incorporated into the blastema. These cells presumably formed a portion of the differentiated tissues of the new regenerate. Studies were performed to verify that the labeled cells in the blastemas were derived from irradiated tissues and were not derived from extraneous sources.

This investigation substantiates reports that irradiation with 2000 R inhibits limb regeneration, and that at least some irradiated cells can contribute to a blastema formed on an irradiated limb.

**INTRODUCTION**

It has been shown that irradiation of the salamander limb with sufficient dosage blocks regeneration and that transplantation of a variety of non-irradiated appendage tissues to the limb reverses the inhibition (skin – Umanski, 1937; Trampusch, 1951, 1958a, b; Rose & Rose, 1965; bone or cartilage – Umanski, 1937; Trampusch, 1951; Stinson, 1968; muscle – Umanski, 1937; Thornton, 1942; Trampusch, 1951; nerve segments – Wallace & Wallace, 1973). If it can be shown that the irradiated tissues do not contribute to the blastema, then all the cells necessary for regeneration may be shown to be derived from any one of a number of diversified tissues. However, if it can be demonstrated that irradiated cells contribute to the blastema, then other means should be used to study the origins and potencies of blastemal cells.

Attempts have been made to solve the problem of participation by studying...
the morphology of the regenerate produced on an irradiated limb. It appears that the non-irradiated graft does control the morphology of the regenerate to a large extent (Stinson 1964a, b; Trampusch 1958a, b), but whether this control is mediated via the contribution of all the blastemal cells by the graft, or is merely a reflection of the loss of morphologic influence of the stump, has not been resolved (see Stinson 1964b; Oberpriller, 1968). Therefore one cannot exclude the participation of at least some irradiated cells on the basis of morphology alone.

Several investigators have tried to approach the problem more directly – that is, by distinguishing in some way between the irradiated tissues and the transplant. Wallace & Wallace (1973) and Tuchkova (1967) used the difference in pigmentation between black and white axolotls to distinguish between irradiated and non-irradiated tissues. Stinson (1964b) used a homograft for the non-irradiated tissue transplant, whereas Namenwirth, as reported by Steen (1970), used tritiated thymidine to label the non-irradiated tissue. These experiments are not conclusive and the reasons are given in the Discussion.

In the present study tritiated thymidine was used to label cells in a limb before irradiation. The movements of the labeled cells were followed when a non-irradiated skin graft was transplanted to the amputated, irradiated limb.

MATERIALS AND METHODS

The right limbs of 85 newts, Notophthalmus viridescens (from Glenn Gentry in Donalson, Tennessee), were amputated half-way between the elbow and wrist. These animals were injected intraperitoneally with 3.0 μCi of tritiated thymidine (specific activity 1.9 Ci/mM, Schwarz Bioresearch) at each of three stages in regeneration: cone, early palette, and early finger-bud.

Of the 85 animals, 65 were chosen for irradiation of the right and left forearms with 2000 R. The irradiations were performed 70 days after the initial amputation of the right limb and approximately 40 days following the last injection. The machine used was a Picker-Zephyr 120, set at 100 kV, 4 mA, no filtration, HVL 0.65 mm aluminium, target distance 20 cm, and dosage 183 R/min. To irradiate several right or left forearms at one time, the animals were anesthetized with MS-222 (Sandoz) and were arranged more or less in a head-to-tail fashion around the periphery of a large Petri dish. The Petri dish was covered with a 0.64 cm lead shield with a hole 2.5 cm in diameter cut in the center. The limbs to be irradiated were arranged so that the elbow and forearm projected into the unshielded area in the middle of the dish (see Oberpriller, 1968).

Operations were performed on the 65 animals 30 days after irradiation. In 40 animals, both the right limb, with the labeled regenerate, and the left limb were amputated through the wrist, stripped of skin from the elbow down, and autografted with shielded hind-limb skin. The right and left limbs of the remaining
25 animals in the irradiated group were merely amputated to find if the 2000 R dose was sufficient to inhibit regeneration.

The left limbs which received grafts were provided to study the contribution of labeled cells from extraneous sources – that is, from the blood, the graft or from incorporation of circulating label. Since the left limbs were not regenerating at the time of the injections of tritiated thymidine, their internal tissues should not be appreciably labeled. The irradiation and grafting procedure should cause these limbs to regenerate at a rate comparable to that of the right. If the blastemas induced on the left limbs contain amounts of label equal to those of the right, then an extraneous source of labeled cells would have to be sought. However, if the left limb blastemas contain considerably less label than those of the right, and, if the possibility of local reutilization of label in the right limb could be eliminated, then one could conclude that the labeled irradiated tissues contributed cells to the regeneration process.

To determine whether there was local reutilization of label, a thorough examination of the epidermis of the right limb grafts was made. Since approximately 70 days were allowed between the last injection and the operations (40 days between last injection and irradiation and 30 days between irradiation and operation), the epidermis of the hind-limb skin should contain no label or only slight label (see Rose & Rose, 1965). If the essentially unlabeled, dividing epidermal cells of the graft did not become heavily labeled at any time during the experiment, then one can assume that there was little, if any, local reutilization.

In addition to the experimentation outlined above, the 20 animals of the original 85 with non-irradiated labeled right limbs were treated in the same way as the irradiated limbs receiving grafts – that is, they were amputated, stripped of skin from the elbow down and were supplied with hind-limb skin grafts. This group was provided to compare regeneration rates between irradiated and non-irradiated limbs subjected to the same traumas. The significance of these results will be discussed later.

Following the operations, each of the newts was kept in a plastic box at 17 °C on paper toweling moistened with the solution used during the operations (1/10 Holtfreter’s plus antibiotics – Rose & Rose, 1965). The animals were removed from the constant-temperature box to room temperature after 4–5 days, and 100 ml pasteurized aquarium water was used in lieu of the operating solution. This water was changed every day for 7 days. After the limb skin graft had healed in place, aquarium water was substituted for the pasteurized water. The animals were fed liver every other day and were maintained individually in their boxes for the remainder of the experiment.

The limbs to be examined microscopically were fixed in Bouin’s fluid and then were decalcified. The tissues were embedded in tissuemat, serially sectioned at 10 μm, and processed for autoradiography by the method of Rose & Rose (1965). The sections were stained with Harris’s haemotoxylin and eosin immediately after development of the emulsion.
Table 1. Observations on regeneration after the operations

<table>
<thead>
<tr>
<th>Amputated limbs</th>
<th>Total</th>
<th>Discards unhealthy</th>
<th>Time in days after operation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Irradiated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>25</td>
<td>4</td>
<td>0/1*</td>
</tr>
<tr>
<td>Left</td>
<td>-</td>
<td>-</td>
<td>0/1</td>
</tr>
<tr>
<td>Irradiated + graft</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>40</td>
<td>6</td>
<td>0/6</td>
</tr>
<tr>
<td>Left</td>
<td>-</td>
<td>-</td>
<td>0/6</td>
</tr>
<tr>
<td>Non-irradiated + graft</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>20</td>
<td>6</td>
<td>0/1</td>
</tr>
<tr>
<td>Left</td>
<td>-</td>
<td>-</td>
<td>0/1</td>
</tr>
</tbody>
</table>

* The numerators represent the number of limbs exhibiting blastemas or differentiating regenerates and the denominators represent the total number of limbs which were observed and fixed at a particular time.

RESULTS

Observations on regeneration after the various procedures

Limbs were removed after the operations at the various times indicated in Table 1. The denominators of the fractions in the table represent the number of limbs fixed at each interval. The numerators represent the number of these limbs which showed signs of regeneration. The irradiated right and left limbs without grafts did not form blastemas, whereas 42% of the irradiated limbs with grafts did.

The results of the grafting operations on the non-irradiated limbs also are shown in Table 1. The rate at which the non-irradiated limbs regenerated was much greater than that of their irradiated counterparts. Normal appearing regenerates with cartilage were produced by 25 days, whereas cartilage appeared only by 44 days in the irradiated group. There is definitely a delay in regeneration and it is assumed that neither the prolonged exposure to tritiated thymidine nor the operation were responsible for it.

Histology of the limbs

Irradiated right limbs without grafts

At 10 and 20 days the epithelium covered the amputation surface of the limbs without grafts. Some labeled cells were being released from the internal tissues at 20 days. Of the limbs fixed at 30 and 37 days, several produced very small accumulations of cells (100–150 cells/section). These cells were very loosely organized. Even by 44 days there was no progress towards blastema formation. The irradiation proved sufficient to block regeneration.
Fig. 1. Section from irradiated right limb regenerate 10 days after receiving a graft. Approximately 20% of the tissues of this limb were labeled. This section is used for orientation for figs. 2 and 3. × 50.

Fig. 2. Labeled muscle cells. × 350.

Fig. 3. Labeled cartilage cells. × 560.

Fig. 4. An irradiated limb 20 days after receiving a graft. This section shows cells accumulating between the wound epithelium and the cartilage. × 70.

Fig. 5. Labeled cells which evidently were released from the cartilage. × 560.
Table 2. The contribution of irradiated cells to the blastema produced on an irradiated limb

<table>
<thead>
<tr>
<th></th>
<th>Blastemal cells/section*</th>
<th>No. of labeled cells/section (4-40 grains) per cell</th>
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<tbody>
<tr>
<td><strong>Irradiated limb + graft</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>30 days</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animal 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>350</td>
<td>27</td>
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<tr>
<td>Left</td>
<td>400</td>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
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<td>14</td>
</tr>
<tr>
<td>Left</td>
<td>500</td>
<td>0, 0</td>
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<tr>
<td><strong>37 days</strong></td>
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<td></td>
</tr>
<tr>
<td>Animal 1</td>
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<td></td>
</tr>
<tr>
<td>Right</td>
<td>850</td>
<td>80</td>
</tr>
<tr>
<td>Left</td>
<td>1000</td>
<td>2, 10</td>
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<tr>
<td>Animal 2</td>
<td></td>
<td></td>
</tr>
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<td>33</td>
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<td>Left</td>
<td>600</td>
<td>1, 14</td>
</tr>
<tr>
<td>Animal 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>550</td>
<td>25</td>
</tr>
<tr>
<td>Left</td>
<td>750</td>
<td>1, 21</td>
</tr>
</tbody>
</table>

* Data in this table were compiled from the average of four non-consecutive sections through the largest areas of blastemal cell accumulations.

Irradiated right limbs with grafts

At 10 days the grafts were well established in three of the six cases studied. The other three grafts were only loosely attached to the internal tissue. The grafts consisted of whole skin and very small bits of muscle. Epidermal migration to cover the wound surface had occurred in all instances, but there was no blastemal-like cellular accumulation in any of the limbs.

The label proximal to the amputation surface of the 10-day limbs was representative of the labeling found in the limbs throughout the series (Figs. 1–3). Four non-consecutive sections from each of the limbs were examined. In each section, the total number of nuclei (excluding blood, dermis and epidermis) were counted in the distal 2 mm, and the number of labeled nuclei were tabulated. 10 % to 30 % of the irradiated, internal cells of these forelimb regenerates were labeled. The label ranged from 4–150 grains per labeled nucleus. Background grain counts were determined by examining areas of the emulsion between the sections. There were rarely three grains over an area the size of the nucleus; therefore, any nucleus with four or more grains was considered to be labeled.

By 20 days the six limbs which were observed showed good graft adherence. Chondroclasts were present and labeled cells, apparently, were being freed from the internal tissues of the limb (Figs. 4–5).
Irradiated cells in regeneration

Figures 6–9

Fig. 6. An irradiated limb 30 days after receiving a graft. There is a sizeable accumulation of cells beneath the apical cap. × 70.

Fig. 7. Labeled cells in the blastema. × 560.

Fig. 8. Section of an irradiated limb 37 days after receiving a graft. There is a very large blastema. × 70.

Fig. 9. Labeled cells in the blastema. × 560.
Fig. 10. An irradiated limb exhibiting cartilage formation 44 days after receiving its graft. × 70.

Fig. 11. One of a few areas of labeled cartilage in the 44-day limb. × 560.

At 30 days two of eight right limbs showed histological signs of early regeneration. An example is shown in Fig. 6. In both limbs the labeled cells were more numerous in the proximal areas of the blastema near the irradiated internal tissues (Fig. 7). The numbers of labeled cells found in these blastemas are given in Table 2. The other limbs in this group exhibited only loose accumulations of cells beneath poor epidermal caps.

Of the eight limbs which were fixed at 37 days, three produced large blastemas (Table 2). One of the three showed especially well the accumulation of labeled cells in the regeneration blastemas and has been chosen to represent the stage (Fig. 8). Again, in the four non-consecutive sections studied in detail, the majority of the labeled cells were found in the proximal portions of the blastemas (Fig. 9), but several also were observed in the distal areas in each section.

By 44 days two of the four limbs had produced regenerates, both of which had cartilage formations (Fig. 10). Very few labeled cells were observed, and these appeared in small pockets in the cartilage (Fig. 11). It was not possible, therefore, to demonstrate a large contribution of irradiated cells to the differentiated tissues of the regenerate. It is interesting to note at this point that the non-irradiated right limb regenerates with cartilage also lacked label, though earlier stage blastemas in this group contained labeled cells. This lack of label in later stages indicates the difficulty of using tritiated thymidine, that is, the label may be diluted to non-detectable levels by cell division or lost with cell death.
**Left limbs**

The irradiated, but not previously amputated, left limbs were treated in the same manner and at the same times as the contralateral right limbs. The irradiated left limbs without grafts did not regenerate, whereas those with grafts did. The blastemas formed at a rate comparable to those of the right (see Tables 1 and 2). The observation of four sample sections of each of the regenerating left limbs revealed, at most, two labeled cells in the blastema per section (5–30 grains). All of the serial sections were scrutinized to be certain that there were no pockets of labeled cells. In contrast to the one or two labeled cells in the left, the average number of labeled cells in the contralateral right blastemas ranged from 14–80 (with 4–40 grains).

**Epidermal label**

The epidermal cells of ten pieces of grafts were examined from samples obtained at the time of the operations. Epidermal cells were also examined in sections of the right limbs with grafts fixed at the various times during the experiment. Counts of 1000 epidermal cells in each graft revealed that 4–5% were labeled at the time of the operations. The average grain count was five grains. (The highest grain count in any one cell was ten grains.) Sections of the right limbs with grafts revealed that the percentage of labeled epidermal cells decreased continually throughout the experiment, and by 44 days, 1% of the cells were labeled with a four grain average. (The highest grain count was five grains.) The epidermal cells counted on the grafted limbs were those located near the junction of the labeled internal tissues and the labeled blastema; yet the percentage of labeled cells and the grain count average decreased. The grain count over any epidermal cell was at no time comparable to the labeling found in the majority of the cells in the right limb blastemas. One can assume that very little, if any, label had been reutilized from the labeled internal tissues of the right limbs.

**Conclusions**

The results from the left limb studies and from the epidermal labeling patterns indicate that the labeled cells observed in the blastemas forming on the irradiated right limbs were derived from irradiated sources. These labeled cells presumably represent only a part of the irradiated cells participating in blastema formation since only 10–30% of the irradiated cells were labeled. A large participation of irradiated cells in cartilage or muscle formation has not been demonstrated, and further research is needed to demonstrate the degree of participation of irradiated cells in tissue formation of the regenerate.
DISCUSSION

It appears from the data presented in this investigation that irradiated cells contribute to the blastema and presumably may contribute to the tissues of the regenerate. This conclusion is supported directly by Tuchkova (1967) and indirectly by Rose, Quastler & Rose (1955). Tuchkova provided evidence suggestive of recovery by using the pigment differences between the black and white axolotls. She grafted a 3–4 mm segment of a black axolotl limb on to the limb stump of a white axolotl. The black graft regenerated a black limb on a white animal. These limb regenerates were irradiated, then amputated. Some were treated with large polymers of RNA; others received no treatment. The treated limbs developed black regenerates in a very small percentage of the cases, but the untreated limbs did not regenerate in a single instance. There were not enough cases, however, to be assured of the results.

Rose et al. (1955), in their study, irradiated forearms of Notophthalmus with 500–10000 R; then amputated the limb through the forearm and stripped the skin to the elbow to allow the migration of non-irradiated epidermis over the stump. The limbs regenerated at all dosage levels, but the size of the regenerate varied inversely with the dose. This result seems to indicate that irradiated cells were participating in the regeneration process. Doses above that which were inhibitory to regeneration of control limbs did not seem to impair the participation of at least some irradiated cells in the regeneration process.

Other investigators have tried to show that irradiated cells do not participate in regeneration. According to Steen (1970), Namenwirth transplanted unirradiated, labeled, tissues to an irradiated limb and found that all the cells in the regenerate were derived from the graft. To my knowledge, the complete data from this quoted experiment have not been published to this time; therefore, one cannot assess their value.

Stinson (1963, 1964a, b) demonstrated through a series of papers that 60-day regenerates, or parts thereof, could produce regeneration of irradiated, partially humerectomized, limbs. The morphology of the regenerates appeared to conform to the implant. To evaluate the origin of the cells of the regenerate, Stinson (1964b) homografted 60-day forelimb regenerates to irradiated arms. After amputation, the limbs produced regenerates which regressed presumably from homograft rejection. Stinson regards the total regression of the regenerates as evidence of non-participation of irradiated cells, but this experiment can be questioned on several grounds. The most important factor is the rapid rate of regeneration. It could be that the rapid regeneration and morphogenesis on the irradiated limb did not allow time for the participation of irradiated cells in the regeneration process. That there is a relatively long delay in the formation of a regenerate on irradiated limbs supplied with more differentiated tissue grafts has been demonstrated in this paper and in experiments reported by Thornton (1942), Trampusch (1951), Rose & Rose (1965) and others. This delay could
allow time for irradiated cells to contribute to the regenerate. A further objection to Stinson’s paper is that there is no assurance that some irradiated cells, which had participated in regeneration, were not victims of generalized tissue destruction in the homograft rejection. It appears to this author that Stinson’s techniques are not refined enough to disprove the participation of at least some irradiated cells.

Wallace & Wallace (1973) reported that the transplantation of segments of peripheral nerves from limbs of white axolotls to amputated irradiated limbs of black axolotls and vice versa resulted in a regenerate which was the color of the graft. The results seem to indicate that many of the cells were supplied by the graft, but one cannot be certain all of them came from that source. Their fig. 1 B shows a black animal with a white graft. It appears that the limb regenerate has at least some pigmentation. This seems to indicate that irradiated tissues were making their way into the regenerate. They also reported an interesting discrepancy in the amount of muscle in the regenerates. Some had more than others. Perhaps this is an indication that irradiated cells were participating more in one limb than in another. This paper, therefore, does not appear to be conclusive.

The present investigation does not completely resolve the controversy of participation. It does indicate, however, that irradiation may be a poor tool for studying the precise origins of cells in the blastema.

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