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Blastema formation and cell division during cockroach limb regeneration

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SUMMARY

Recent models of pattern formation in insects have been derived largely from observations on regenerated cuticular patterns. Such models make assumptions about the behaviour of the underlying epidermal cells, their movement and patterns of cell division. The present study, designed to test these assumptions, looks at the patterns of wound healing and cell division after amputation at the trochanter–femur joint of the metathoracic leg in the cockroach. It shows that the wound is closed by cell migration and that regeneration occurs by dedifferentiation of the trochanter and distal coxa to form a blastema which grows and redifferentiates to form the new limb. The extent of the spread of dedifferentiation is confirmed by a scanning electron microscope study of the coxa after the moult following amputation. The results highlight the need for a greater knowledge of cell behaviour during pattern formation before we can begin to understand the processes involved in pattern formation.

INTRODUCTION

Regeneration in insects has been studied in two main ways. The majority of studies concerned with pattern formation have concentrated on the cuticle and attempted to deduce the underlying cellular processes that create the altered cuticular patterns observed. Such studies have led to the concept of positional information (Wolpert, 1971). The nature of positional information is as yet unknown. The use of the word 'gradient' to describe the changes in positional value has suggested the idea of a chemical gradient of a diffusible morphogen, and a number of models have been suggested to explain gradient behaviour in terms of production, diffusion and breakdown of diffusible substances (e.g. Gierer & Meinhardt, 1972). One requirement of all such models is the existence of boundaries. For a long time it was thought that segmental borders provided boundaries. However, it has recently been shown that segmental borders can be intercalated (Wright & Lawrence, 1981) and are therefore not true boundaries. Similar results to those of the insect segment have been found by grafting around the circumference of the limb (French, 1978). Here it has been shown that there are no boundaries and that the intercalated values will take the shortest route around the limb. This has led to the formulation of a model based on local cell

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to cell interaction known as the 'polar coordinate model' (French, Bryant & Bryant, 1976, since revised Bryant, French & Bryant, 1981). The requirements of this model are that regeneration is epimorphic and that during limb regeneration positional values are laid down in a proximodistal order with cell division occurring at the tip of the regenerating limb.

The other approach to the study of regeneration has been to look at tissues, particularly the epidermis, during the course of regeneration. Wigglesworth has studied the behaviour of the epidermis in *Rhodnius*, including its regeneration after wounding (Wigglesworth, 1937), and Bohn (1976) has looked more closely at the process by which the epidermal cells migrate to cover the wound.

The present study seeks to relate these two approaches to gain a clearer overall picture of what actually happens when insect tissues are regenerated. This paper looks at the behaviour of epidermal cells during regeneration from the preformed breakage plane between the trochanter and femur of the hind leg in the cockroach, *Periplaneta americana* (Fig. 1). Tritiated thymidine and colchicine are used independently to study patterns of cell division, and scanning electron micrographs are used to show the altered cuticular patterns in the regenerated limbs. These indicate how much of the non-amputated tissue is involved in the regeneration. The results show that, after the wound has healed by cell migration, a blastema is formed by dedifferentiation of the surrounding epidermis. The blastema then grows and redifferentiates to form a new limb. This rules out the possibility that regeneration is completely epimorphic.

MATERIALS AND METHODS

Stocks of *Periplaneta americana* were kept at 24 °C and fed commercial rat food and water. Oothecae were removed, and checked each day for newly hatched larvae, which were kept until the day after their first moult (about 12 days). Operations were performed after anaesthetizing with CO_2 and consisted of gently pulling the left metathoracic limb to separate it at its preformed breakage point.

In order to show the distribution of cell divisions at different stages of regeneration, animals were either injected 12 h before fixation with a 1 % colchicine solution (0·5 μl per 0·1 g live weight of animal) made up in Clarke's insect saline (Hale, 1965), or, 4 h before fixation, with tritiated thymidine (37 MBq ml^{-1} injected at 2 μl per 0·1 g live weight of animal). The parts of the legs to be studied were fixed in a glutaraldehyde/paraformaldehyde mixture (Karnovsky, 1965) buffered in a phosphate buffer (Hayat, 1970) at pH 7·4, for 3 h, then dehydrated either through an acetone series and embedded in Araldite, or through an alcohol series and embedded in LR White (London Resin Company). Semithin sections (1·5 μm) were cut, using a Huxley Ultramicrotome with glass knives, and stained with either toluidine blue or with Lee's methylene blue/basic fuchsin stain (Bennett, Wyrick, Lee & McNeil, 1976). Sections were

photographed on a Zeiss Photomicroscope II. Autoradiography was carried out prior to staining. Slides were coated with NTB-2 emulsion (Eastman-Kodak) and stored at room temperature for 5 weeks before being developed in Kodak D-19 developer and stained with methylene blue.

Material for the scanning electron microscope (SEM) was fixed as for sectioning, dehydrated through an acetone series, critical-point dried in CO_2 using a Tousimis Samdri-780 critical-point drier, coated with a 1·5 nm layer of gold using a Polaron sputter coating unit and examined with an ISI-60 SEM.

Whole mounts of legs for light microscope study of the bristle patterns were

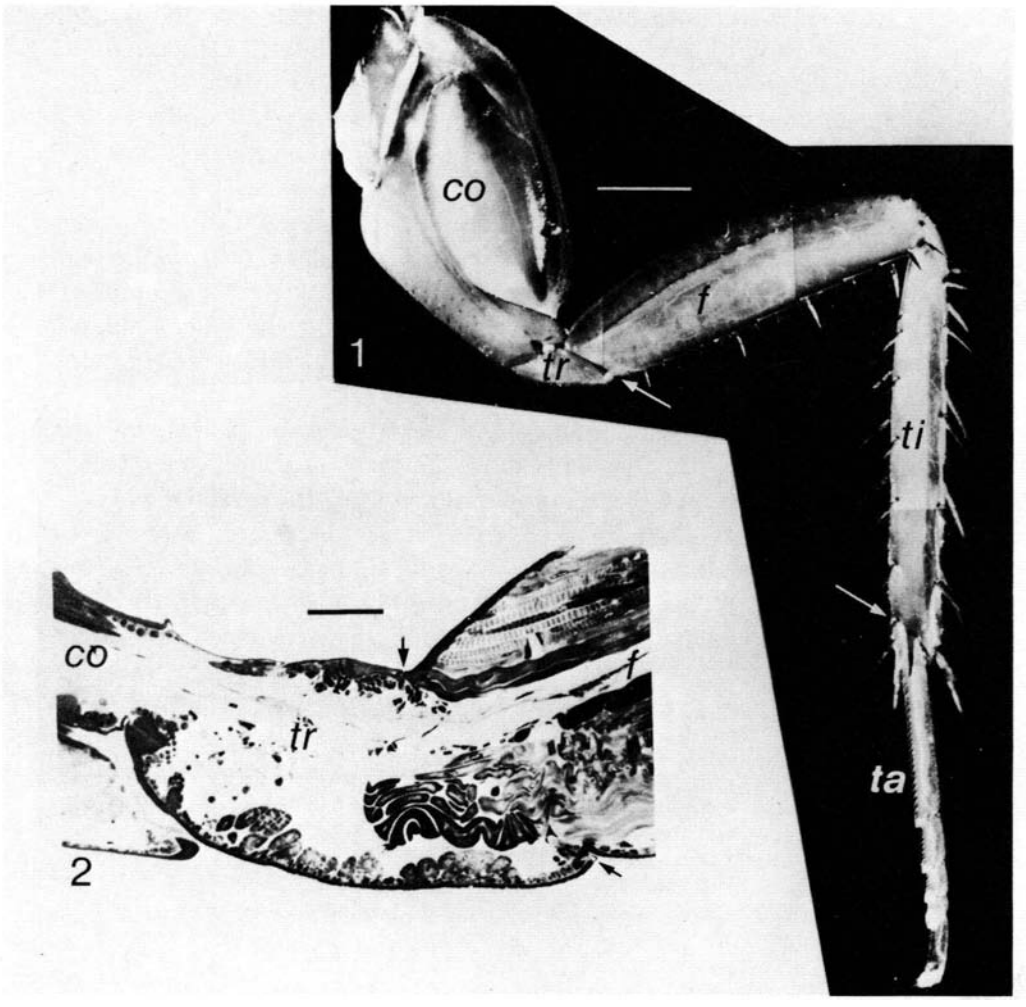

Fig. 1. Left metathoracic leg showing segmentation and preformed breakage planes (arrowed). co, coxa; tr, trochanter; f, femur; ti, tibia; ta, tarsus. Scale bar 500 μm.

Fig. 2. 1·5 μm resin section through the trochanter, showing the location of thin points in the cuticle at the breakage plane (arrowed). co, coxa; tr, trochanter; f, femur. Scale bar 100 μm.

prepared by boiling in 10% KOH for 5 min, leaving to stand overnight, dehydrating in alcohol and mounting in neutral mounting medium.

Mitotic frequencies were calculated in each section as average number of mitotic figures per 100 μm of epidermis.

RESULTS

Morphology

The observed changes in morphology that occur during limb regeneration from the trochanter–femur preformed breakage plane agree with those made from whole mounts by Bodenstein (1955) and Penzlin (1963). However, the 1·5 μm resin sections permit a more detailed description of the histological changes that occur in the epidermis. The process of regeneration can be divided into three parts: wound closure, blastema formation, and blastema growth and differentiation.

Figs 2 and 7 show a section through the trochanter. The joint with the coxa is flexible and can be moved by the large muscles within the coxa, whereas the joint with the femur is rigid and any attempt to move it causes the femur to break off at the thin point in the cuticle. The muscles which pass through this joint and which move the femur tibia joint also snap, and the stumps which are left in the trochanter break down within about 30 h (Fig. 8).

The process of wound closure in insects has been described by Wigglesworth (1937) and Bohn (1976). The closure across the preformed breakage plane is consistent with their descriptions. Soon after amputation, the wound is sealed by a clot of haemolymph which hardens to form a scab. Large numbers of haemocytes congregate underneath the scab, those closest to it taking on a flattened appearance. At the same time, the epidermal cells to either side of the wound become enlarged and their nuclear material, particularly the nucleolus, becomes more distinct (Fig. 8). Cells in this stage were described by Wigglesworth as 'activated'. These processes are completed in about 24–30 h in second

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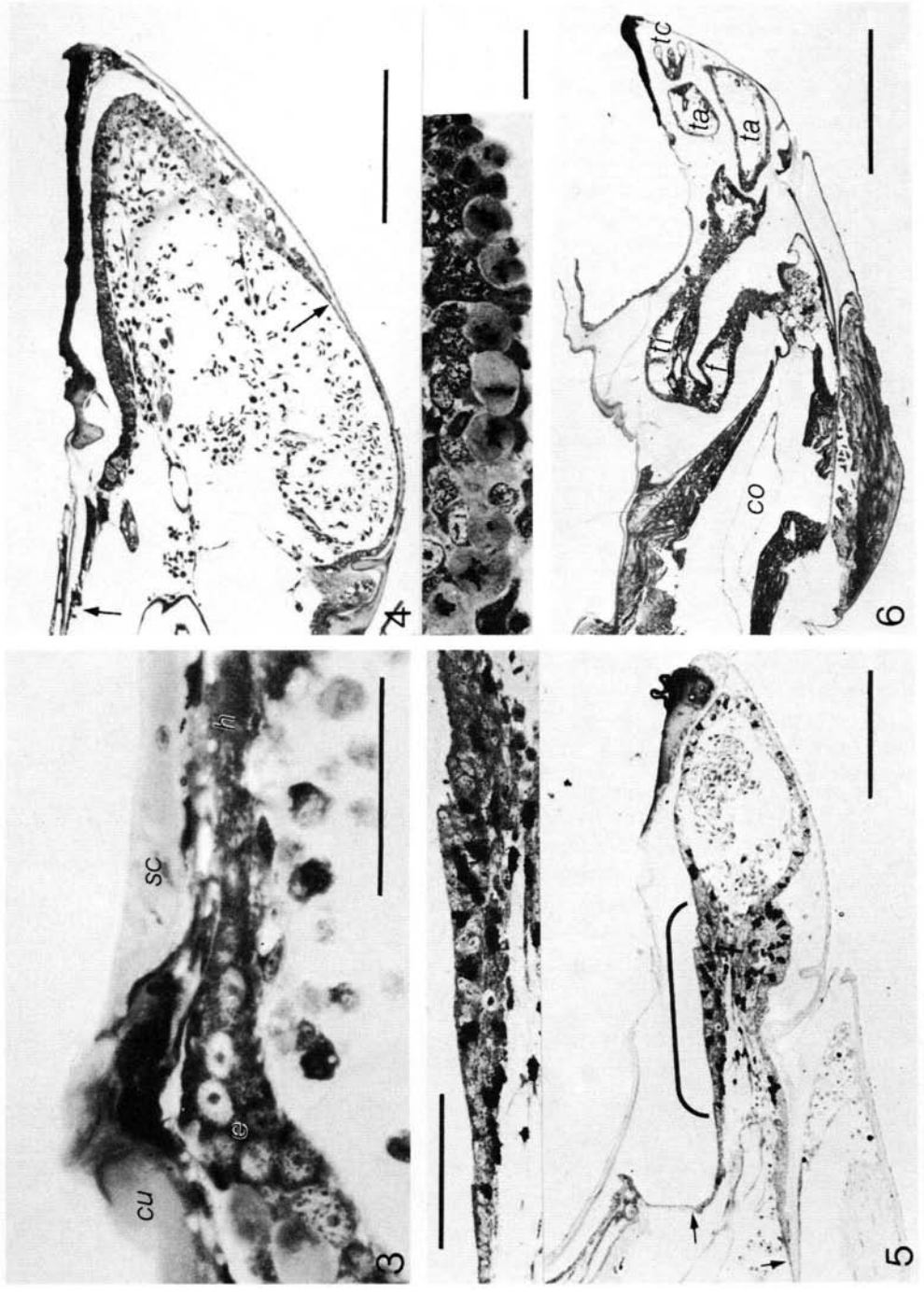
Figs 3–6 1·5 μm resin sections through regenerating legs.

Fig. 3. Edge of wound area 1½ days after amputation with epidermal cells starting to migrate across the wound. cu, cuticle; e, epidermis; sc, scab; h, flattened haemocytes. Scale bar 100 μm.

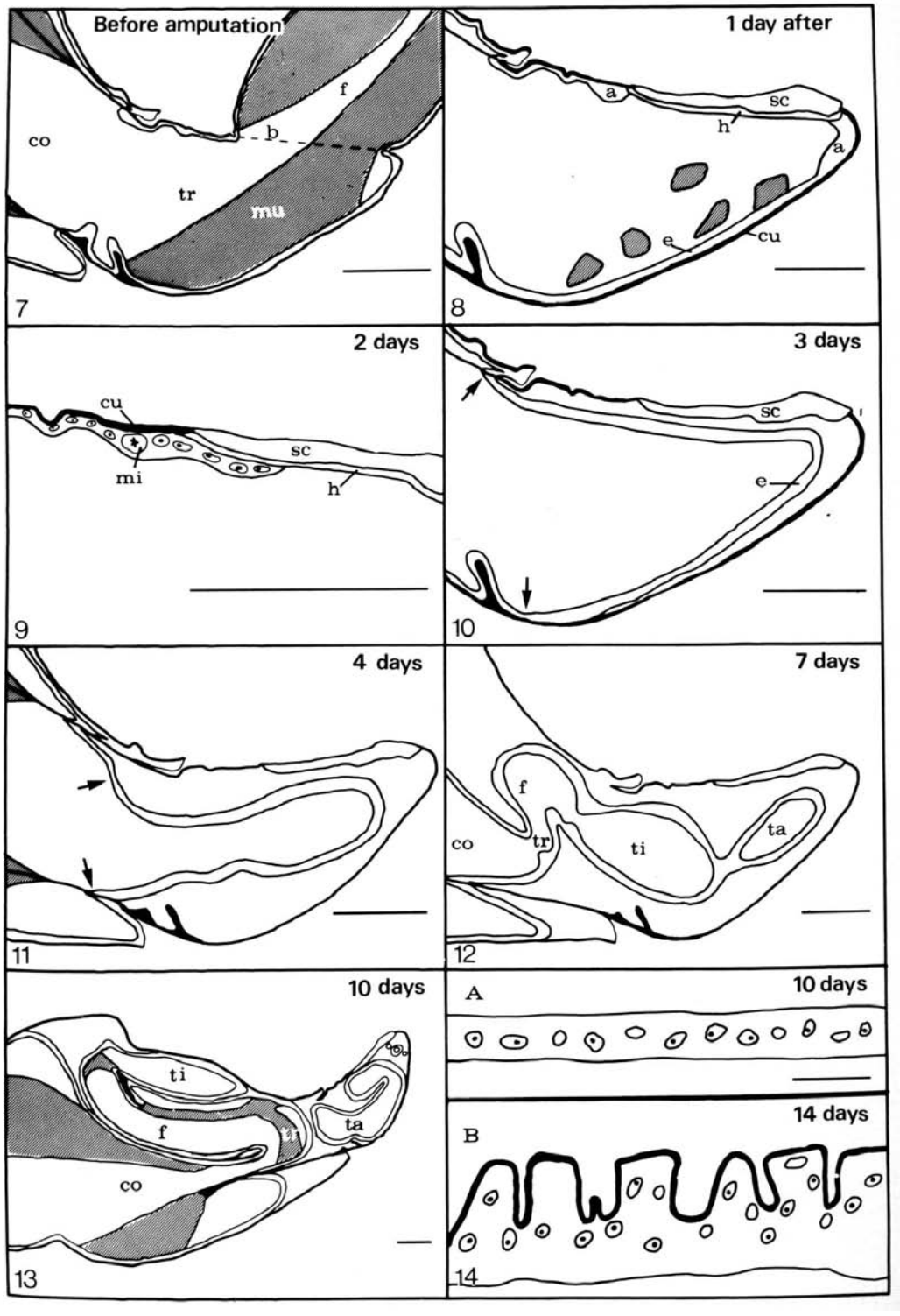
Fig. 4. Whole trochanter 3 days after amputation. Wound healing complete and cells dividing in activated area around the wound. Limit of spread of activation arrowed, and mitoses arrested over 12 h with colchicine. Scale bar 200 μm. Inset shows detail of arrested mitoses. Scale bar 20 μm.

Fig. 5. 4 days after amputation. Autoradiographs after a 4 h pulse of [^{3}H]thymidine, showing band of high DNA synthesis (bracketed) and limit of spread of activation (arrowed). Scale bar 200 μm. Inset shows details of band. Scale bar 100 μm.

Fig. 6. 8 days after amputation. Regenerating leg has segmented and is growing, folded within the cuticle of the coxa and trochanter. co, coxa; tr, trochanter; f, femur; ti, tibia; ta, tarsus; tc, tarsal claws. Scale bar 500 μm.



Figs 3–6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Figs 7–14

instar larvae, after which the activated epidermal cells start to migrate across the wound following the line of the underside of the flattened haemocytes (Figs 3, 9). As migration proceeds, the activation spreads outwards to cover a larger area of epidermis, and the activated epidermis pulls away from the cuticle. After about 3 days, the migrating epidermal cells meet and epidermal continuity is restored (Figs 4, 10).

In wound healing after cuts and burns, cell density is restored by cell divisions to either side of the wound during and immediately after migration, and the cells then return to the resting state. In limb regeneration, however, activation and separation of epidermis from cuticle continue to spread outwards to include cells from the whole of the trochanter and also the distal end of the coxa (days 4–5), thus forming a blastema from which the new limb will grow (Figs 5, 11). It is difficult to determine from direct observation exactly how far back the activation spreads, as growth of the blastema pushes activated cells further back into the coxa, but it appears to be about 600 μm or 55 cell lengths in a 2nd instar animal.

Growth of the blastema is initially very rapid, and by day 7 the regenerating limb has started to fold back into the coxa (Fig. 12). At the same time that it is growing, the limb is also redifferentiating. By day 5 reproducible folds in the blastema show the start of segmentation, which is completed by day 7. By the end of day 6 developing tarsal claws are clearly visible and these are followed by the spines on the tibia and femur. New muscles can be seen forming from day 6, and they continue growing until the end of the moult cycle. Although their origin is not obvious, they appear to form from clusters of myoblasts within the haemocoel. By day 13 the epidermis of the regenerate has become extensively folded and about this time apolysis occurs, and a new cuticle is formed (Fig. 14). On about day 16, the animal moults and the regenerated limb expands and unfolds, presumably under hydrostatic pressure.

Figs 7–14. Histology of regenerating limb. co, coxa; tr, trochanter; f, femur; ti, tibia; ta, tarsus; mu, muscle; b, breakage plane; e, epidermis; cu, cuticle; mi, mitotic figure; sc, scab; h, layer of flattened haemocytes; a, activated epidermis. Arrows show limits of spread of activation. Scale bars: Figs 7–13, 200 μm; Fig. 14, 20 μm.

Fig. 7. Before amputation.

Fig. 8. 1 day. Clot and scab formed, epidermis becoming activated, and muscles breaking down.

Fig. 9. 1½ days after amputation. Epidermal cells migrate along underside of flattened haemocytes. Some cells start to divide to either side of the wound.

Fig. 10. 3 days after amputation. Activation has spread outwards and the epidermis is becoming detached from the cuticle.

Fig. 11. 4 days after amputation. Blastema formation is almost complete.

Fig. 12. 7 days after amputation. Regenerating limb segmented and folding back into coxa.

Fig. 13. 10 days after amputation. Growth almost complete and new muscles formed.

Fig. 14. Between days 10 (A) and 14 (B) the epidermis becomes folded and the new cuticle is formed.

Patterns of cell division as shown by colchicine

A 12 h exposure to colchicine resulted in a high proportion of arrested divisions in each section, enabling their distribution to be seen clearly.

During wound closure, very little cell division occurs. However, the little that does take place is mainly in the region just to either side of the wound (Figs 3, 15) as one would expect from Wigglesworth's (1937) observations. As activation spreads outwards from the wound, so the activated cells start dividing, although initially few of those cells that have migrated to close the wound divide (Fig. 16). By the end of day 4, when the blastema has formed, cell divisions are occurring very rapidly throughout its epidermis (Fig. 17). Cell divisions are so numerous at this stage that during the 12 h exposure to colchicine, most of the cells have divided and some would have divided twice if they hadn't been arrested at the first division. This gives a distorted result for the differential rates of cell division. For this reason days 4 to 7 were repeated using a 4 h exposure. From these it was possible to show that cells reach a maximum rate of division soon after they become activated. Thus a band of high mitotic activity is seen behind the spreading activation front (days 4 and 5, Figs 18, 19). As mitotic activity spreads into the wound area another peak of mitotic activity is formed there (day 4, Fig. 18). As the blastema grows, mitotic figures first become evenly distributed (days 7 to 8) and then their frequency decreases (days 8–12), with the level falling first in the tarsus. On day 9 the frequency in the tarsus is half that on day 8, on day 10 a quarter and on day 12 an eighth. However the frequency in the tibia and femur starts to decrease on day 10, falling to about a quarter of its day 10 level by day 12.

The formation of new muscles also involves extensive cell divisions. At about day 10, cell divisions occur in epidermis of the coxa which has not been incorporated into the blastema. This corresponds to the normal pattern of cell division during intermoult growth (Fig. 21). All epidermal cell divisions cease by day 13

Figs 15–22. Patterns of cell division as shown by colchicine injections. Figs are drawn from single sections, the animal being fixed 12 h after injection, except for Fig. 18, which was compiled by adding together 3 sections from a 4 h exposure. sc, scab; mi, mitotic figures; cu, cuticle; e, epidermis; co, coxa; tr, trochanter; f, femur; ti, tibia; ta, tarsus; mu, muscle. Scale bars, 200 μm.

Fig. 15. 1½ days after amputation. Mitotic figures to either side of wound.

Fig. 16. 3 days after amputation. Mitotic figures spreading out from wound area.

Fig. 17. 4 days after amputation. Blastema saturated with mitotic figures.

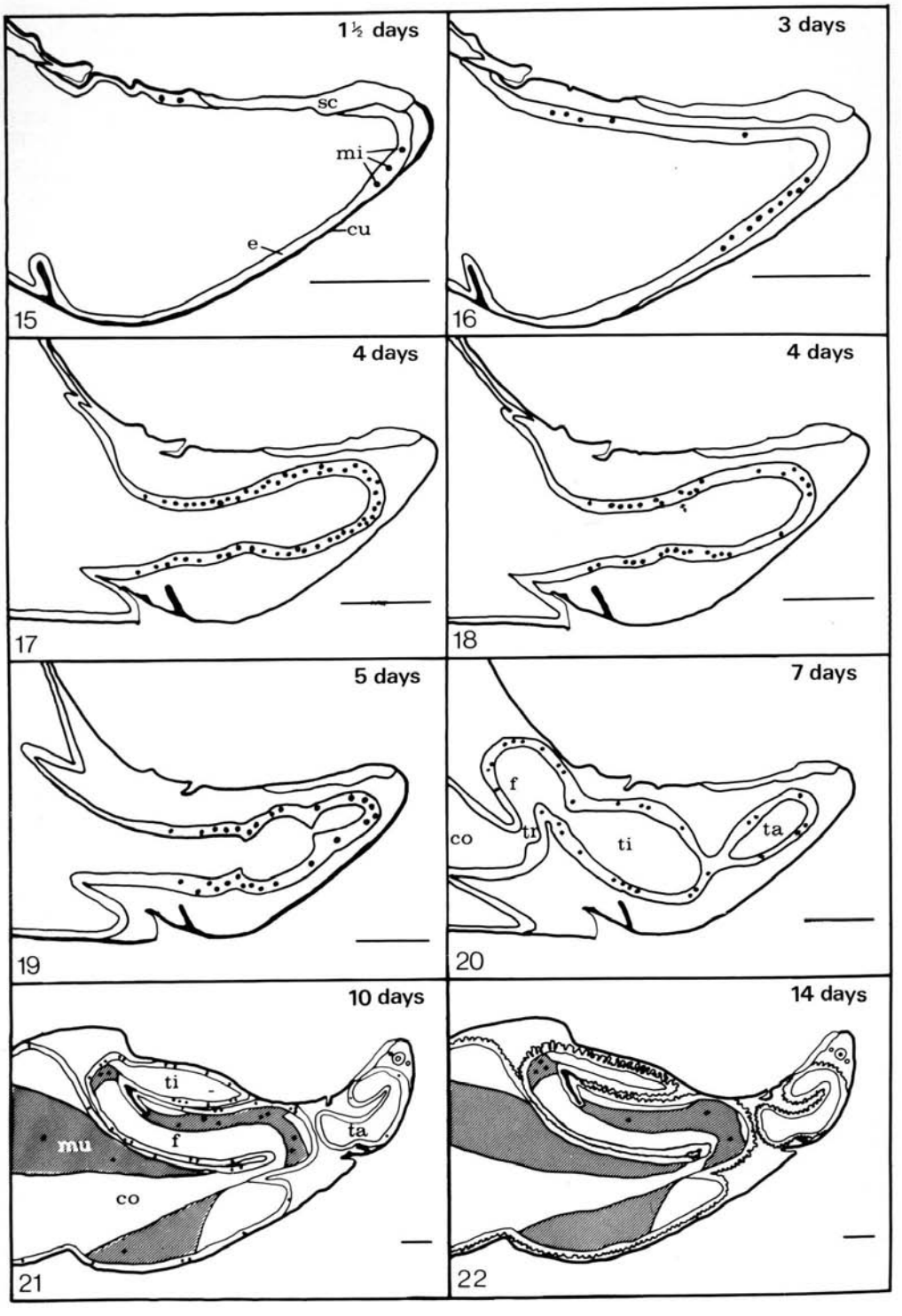
Fig. 18. 4 days after amputation. A shorter exposure to colchicine shows an area of high mitotic activity behind the activation front with a lower activity at the distal end (see text).

Fig. 19. 5 days after amputation.

Fig. 20. 7 days after amputation.

Fig. 21. 10 days after amputation. Mitotic figures in developing muscles and in epidermis and muscle of coxa.

Fig. 22. 14 days after amputation. Mitotic figures only in regenerating muscles.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Figs 15–22

just prior to cuticle secretion. However, divisions continue in the new muscles right up until ecdysis (Fig. 22).

Patterns of DNA synthesis after amputation as shown by [^{3}H]thymidine

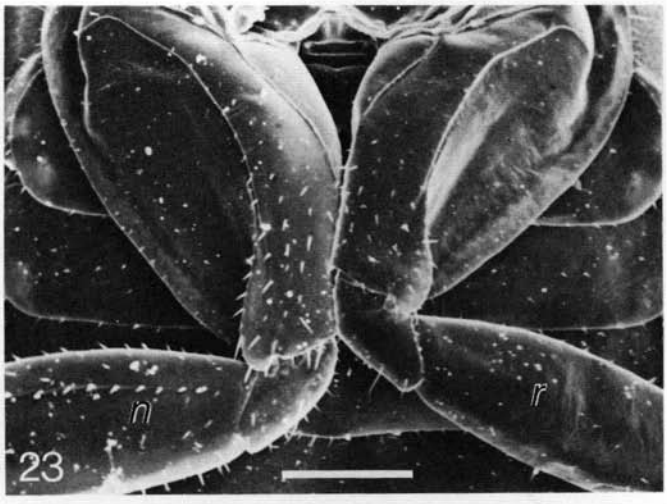
[^{3}H]thymidine is incorporated into replicating DNA and hence labels an earlier stage of cell division than colchicine. The patterns shown in the autoradiographs are similar to those shown by colchicine. The label appears first in the nuclei of cells adjacent to the wound area on day 2, then spreads into the wound area and outwards, following the spread of activation, but preceding that of mitotic activity. The band of rapid DNA synthesis behind the activation front is much steeper than that for mitotic activity (Fig. 5) with very little synthesis occurring at the distal end of the blastema after day 4. After segmentation (day 6–7) nearly all the DNA synthesis occurs in the femur and tibia, until day 9 when epidermal DNA synthesis decreases and stops by day 12. As with mitotic figures DNA synthesis is seen in the developing muscles right up to the end of the moult cycle.

Changes in cuticle pattern

After amputation the regenerated segments are shorter than in a normal leg. In some cases the coxa of the regenerated leg appears to be smaller than that of the control leg (Fig. 23). The reason for this is uncertain, but it could be that the regenerating leg in some way inhibits the normal growth of the coxa between second and third instars. This effect is variable from animal to animal and is often negligible. It probably has nothing to do with remodelling the coxa to form the blastema. A reliable indication for regenerated tissue is the distribution and size of spines and bristles. In parts of the leg that are clearly regenerated they are generally reduced in size and number (Fig. 23). This means that, by looking at the cuticle immediately proximal to the level of the wound, it is possible to identify which structures have been regenerated and hence how far back the dedifferentiation has spread. Bristles on the trochanter of the regenerated leg are always reduced in size and number, whereas those over most of the coxa are the same on both the control and regenerated legs (Fig. 24). The distal end of the coxa was surveyed for marker bristles which could be used to establish how much

Fig. 23. Scanning electron micrograph of the coxa, trochanter and proximal femur of a normal (n) and a regenerated (r) leg. The reduced bristle density and size on the femur and trochanter is characteristic of newly regenerated leg tissue. Scale bar 500 μm.

Fig. 24. Enlargement of coxa–trochanter joint of the specimen illustrated in Fig. 23 showing that bristles on the proximal coxa of the regenerated leg (right) are similar in size and density to those of the control. Bristles along the distal edge of the coxa are always reduced in size following regeneration. The bristle arrowed is also reduced in size in 50 % of regenerated legs and therefore marks the region of distal coxa which is involved in the remodelling process. Scale bar 200 μm.



Figs 23–24

of the coxa is remodelled during regeneration. The anterior face of the coxa provided the only usable markers because near the end of the coxa, the posterior face is largely devoid of bristles. Bristles along the distal anterior margin of the leg are arranged in a predictable pattern and are always reduced in size (approximately half as long) following regeneration (Fig. 24). Immediately proximal to this level on the coxa there are no large identifiable bristles. However there is one 120 μm from the edge of the coxa (arrowed in Fig. 24). This bristle was examined in regenerated and control metathoracic legs of eight animals (four S.E.M. preparations and four light microscope preparations). It was reduced to about a half normal size in four regenerated legs compared with the controls. In the other four animals the bristle was either slightly reduced or not reduced at all. Since the bristle had regenerated in at least four out of eight cases it suggests that this bristle lies close to the proximal limit of the tissue that is involved in the regeneration process.

DISCUSSION

Regeneration of the cockroach limb appears to involve three main stages. Firstly the wound is healed in a manner similar to that following a simple cut in the cuticle and epidermis (Wigglesworth, 1937). During this time, cell divisions occur in the activated epidermis to either side of the wound. It has been shown that activation and migration can be induced by polypeptide products of damaged cells (Wigglesworth, 1937) and that cellular depletion, such as is caused by the migration of cells towards the wound can be responsible for mitotic activity. In a normal wound the epidermis will return to its resting state, once continuity and cell density have been restored. However, after limb amputation, the cells remain activated and activation and mitotic activity spread outwards from the wound to incorporate the epidermis of the whole of the trochanter and the distal end of the coxa into a blastema from which the new limb develops. This conclusion disagrees with that of Penzlin (1963), who suggested that the blastema formed entirely from the epidermis in the immediate vicinity of the wound, as would be required by the Polar Coordinate Model. Similar results to those described here were found by Bullière (1972) who looked at tarsal regeneration and who also concluded that blastema formation involved respecification of the surrounding epidermis rather than division of the cells adjacent to the wound area.

After the initial spread of activation leading to blastema formation, the position of the base of the blastema remains the same during its growth and redifferentiation, with no further noticeable spread of activation.

The first signs of redifferentiation are constrictions in the epidermis, which form the first joints, and the clustering of myoblasts at the sites of the future muscle insertions. By studying legs between days 6 and 8 of regeneration it appears that the first two joints formed are those between the femur and tibia and

the tibia and tarsus, with the coxa–trochanter and trochanter–femur joints forming later. This would suggest a distoproximal order of redifferentiation rather than the proximodistal order suggested in the past (O'Farrell & Stock, 1954). Further support for distoproximal redifferentiation is that during the early stages of regeneration, cell division occurs in what will be the distal end of the leg, whereas later on it is the proximal segments that show a higher rate of cell division.

Conclusions

Regeneration of the cockroach leg from the trochanter–femur breakage plane requires respecification of the trochanter and distal coxa, and this appears to occur in a distoproximal sequence. One might expect that the same type of process could be involved in intercalation after grafting experiments. But these generally produce rather little new epidermis compared with a complete new leg, and it appears that the spread of activation depends on the amount of new tissue to be produced. If only the tarsus is amputated, its regeneration requires a spread of activation of only about 150 μm, as compared with 600 μm for regeneration from the trochanter–femur joint (Truby, unpublished data). Two grafts that might require enough intercalation to show a spread of activation are either when proximal and distal levels of a leg segment are grafted together, or where the leg's axes are reversed at the graft, resulting in the production of supernumerary legs (Bohn, 1965). These situations are being studied and the results will be reported later.

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