The growth of supernumerary legs in the cockroach

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SUMMARY

When the anteroposterior axis of a cockroach leg is reversed at a graft by exchanging a left leg for a right leg at the mid-tibia level, regeneration occurs in the region of the graft/host junction. This results in the formation of a pair of lateral supernumerary legs. In these experiments the patterns of cell division which take place during supernumerary leg formation were observed in sections of regenerating legs of the cockroach Leucophaea maderae. Early patterns of cell division resemble those seen in control grafts in which no axial reversal had been carried out during grafting. These cell divisions are associated with the process of wound healing. Later, a large area of the epidermis proximal to the graft/host junction becomes activated and shows a rapid rate of cell division. This area forms two outgrowths which grow by cell division throughout their epidermis to form the epidermis of the supernumerary legs.

The results are more consistent with the view that the formation of supernumerary legs involves dedifferentiation of the epidermis in the region of the graft/host junction to form a blastema, rather than being due to local cell division at the point of maximum pattern discontinuity. This conclusion is used to offer an explanation for the range of different types of outcome of left–right grafts that has been observed.

INTRODUCTION

Grafts in which a donor left leg, amputated at the mid-tibia level, is grafted onto a host right leg, amputated at the same level, have been described for a number of cockroach species (Leucophaea maderae, Bohn, 1965; Blaberus craniifer, Bullière, 1970; Blattella germanica, French, 1976b). In most of the cases where the external and internal faces have been lined up (leaving the anterior face of the host in contact with the posterior face of the graft and vice versa) the outcome is the formation of a pair of supernumerary legs at the graft/host junction (Fig. 1). By using light and dark body colour mutants of Blattella (French, 1976b) or different cockroach species (Bohn, 1972) for the host and graft it has been shown that the epidermal tissues of the supernumeraries are composed half of host origin and half of graft origin, with the graft-derived tissue forming the distal side of each supernumerary.

These results have been explained in terms of local cell division stimulated by the pattern discontinuities between the opposing anterior and posterior faces (e.g. Bryant, French & Bryant, 1981). Consecutive rounds of cell division are assumed to occur at the points of maximum discontinuity between the graft and host tissue. These produce progressively more distal levels of the leg, until the distal tip is reached.

Key words: cockroach, supernumerary legs, blastema formation.
During cockroach leg regeneration it has been shown that the regenerated tissue is not produced by local cell division at the tip of the regenerating leg, but involves dedifferentiation and respecification of tissue proximal to the wound level (Bullière, 1972; Truby, 1983a).

This paper looks at the epidermal changes and patterns of cell division that occur during the formation of supernumerary legs. It shows that, as for leg regeneration, division of epidermal cells occurs over a considerable area surrounding the points of maximum pattern discontinuity. It is suggested that this is more likely to be the result of a spreading of activation and cell division into the surrounding epidermis than of purely local division of cells from the graft/host junction.

MATERIALS AND METHODS

Stocks of *Leucophaea maderae* were kept at 24°C and fed commercial rat food and water.

Operations were performed on recently moulted animals. Sixth instar larvae were used as hosts and fourth instar larvae as donors. The animals were first anaesthetized by placing them in a container surrounded by ice for half an hour. For experimental grafts, the left metathoracic leg of the donor animal was amputated at the mid-tibia level using a mounted piece of razor blade, and the right metathoracic leg of the host was amputated just distal to this level. The graft leg was then rotated through 180° to line up its internal and external faces with those of the host (making the anterior and posterior faces out of register) (Fig. 1), and inserted into the stump of the host leg (with a small overlap to hold it in position). The graft was sealed in place using molten insect wax (Krogh & Weiss-Fogh, 1951). For control grafts, left legs were grafted into left stumps.

In order to show the distribution of cell divisions at different stages of regeneration, animals were injected 12 h before fixation with a 1% colchicine solution made up in Clarke's insect saline (Hale, 1965).

The parts of the legs to be studied were fixed for 3 h in a glutaraldehyde/paraformaldehyde mixture (Karnovsky, 1965) buffered in a phosphate buffer (Hayat, 1970) at pH 7.4, then dehydrated through an acetone series and embedded in Araldite or Spurr's resin. Semi-thin sections (1.5 µm) were cut in either the longitudinal plane of the leg, parallel to the internal–external axis as this was found to show the developing outgrowths most clearly (Fig. 2), or in the horizontal plane to show the complete circumference of the leg. Sections were stained with toluidine blue and mounted in Araldite.

To show the distribution of mitotic figures a 1.5 µm section was cut and mounted every 10 µm over as much of the thickness of the leg as possible for longitudinal sections (Fig. 3) or from about 400 µm proximal to 200 µm distal to the graft/host junction for horizontal sections. For longitudinally sectioned legs the distribution of mitotic figures above and below the graft/host junction was calculated and plotted as shown in Fig. 3 (the junction in grafted animals was taken as the proximal limit of graft cuticle where it overlapped with the host and for unoperated controls the centre of the scored region was taken as the midpoint of the tibia). For unoperated controls and control grafts the values from a number of different legs were combined to give the final values for the graph. In experimental grafts the degree of circumferential pattern discontinuity between the graft and host varies around the circumference (greatest at the anterior and posterior faces, least at the internal and external faces), and circumferential differences in the patterns of cell division were expected. For this reason the internal and external sides of the leg were plotted separately. Where a region of high mitotic activity was found around the level of the graft, the values were replotted to indicate the circumferential distribution of mitotic figures forming the region of high mitotic activity.

For horizontally sectioned legs the proximodistal band of high mitotic activity was found from the total number of mitotic figures in each section. The epidermis of sections within this band was divided into 24 equal lengths around the leg and the number of mitotic figures in each length was counted and divided by the length of the epidermis around the circumference of the leg to
RESULTS

Unoperated controls

In unoperated legs of sixth instar animals, cell division is negligible until the 12th day of the moult cycle. After this time it becomes very variable. On the 17th day the mean value for an animal was found to vary from 0.02 to 0.40 mitotic figures per 100 μm of epidermis (based on ten sections from each of five animals), with a mean of 0.25 mitotic figures per 100 μm. This variability is probably due to differences in moult cycle length, some animals having reached the normal time for intermoult growth and others not. At all times mitotic figures appeared to be distributed randomly throughout the epidermis of the leg.

Control grafts

The sixth instar host and fourth instar donor were chosen so that when the donor tibia was inserted into the host tibia, the fit would be as close as possible. However,
there was always a slight gap left between the graft cuticle and the host epidermis. The result of this is that during wound healing, the epidermal cells have to migrate over the outer surface of the graft cuticle, for a distance that varies from one graft to another (Fig. 4). This probably means that the time taken for the completion of wound healing and the restoration of epidermal continuity is also variable. This in turn may contribute to the variations seen at later stages.

Fig. 2. The planes for sectioning. Most of the outgrowths that were left to develop fully formed towards the internal or external faces of the leg. These are most clearly shown in sections cut parallel to the internal–external plane and between lines C and D to include a single layer of epidermis on either side of each section. Sections parallel to the anteroposterior plane would have to lie between lines A and B and would not include the outgrowths. To show the whole circumference some legs were sectioned horizontally (in the plane of the page). cu, cuticle; sp, space where the epidermis has separated from the cuticle; e, epidermis; o, outgrowths.

Fig. 3. Plotting mitotic figures in longitudinal sections. (A) The distribution of mitotic figures in each leg is obtained from 24–30 sections taken 10 \( \mu \)m apart along the anteroposterior axis (i). In each section the epidermis is divided into 100 \( \mu \)m lengths for a distance of 1000 \( \mu \)m to either side of the graft/host junction (ii). The average number of mitotic figures in each 100 \( \mu \)m length of epidermis is calculated by combining the figures for equivalent 100 \( \mu \)m lengths of each of the sections. These figures are used to plot the proximodistal distribution of mitotic figures along the internal and external sides of the leg (iii). For control legs the values for the internal and external sides are combined and averaged to give an overall proximodistal distribution (see Fig. 6). (B) For experimental animals a circumferential distribution of mitotic figures is also plotted for the epidermis within the band of high mitotic activity. This is done by calculating the average number of mitotic figures per 100 \( \mu \)m within the band along the internal and external sides of individual sections (locations of sections around the circumference shown in (iv)). These values are plotted to show the circumferential distribution of mitotic figures within the band of high mitotic activity (v).
On completion of wound healing, the epidermis to either side of the graft/host junction starts to separate from the cuticle to a distance not usually more than 50–100 μm from the junction. At the same time the epidermal cells around the level of the junction appear ‘activated’ (enlarged, with enlarged nucleus and nucleolus, Wigglesworth, 1937) and start to divide (Fig. 5). The signs of activation
are less striking than has been noticed previously during leg regeneration from the trochanter–femur joint (Truby, 1983a), but an overall thickening of the epidermis due to enlargement of the epidermal cells is always clearly visible. Cell division continues until about 20 days after grafting, after which time apolysis throughout the leg occurs, the epidermis becomes highly folded and a new cuticle is secreted.

Fig. 6 shows the proximodistal distribution of mitotic figures for control grafts. After 5 days there is a band of high mitotic activity at the level of the graft/host junction. To either side of this are regions of lower mitotic activity extending 500 μm to either side of the junction. The average mitotic frequency in these regions of lower mitotic activity is 0.08 mitotic figures per 100 μm (about 27 times the frequency in ungrafted legs at this time). After 10 days the central band has a lower mitotic frequency, but the width of the activity to either side has increased to cover the whole 2000 μm length of epidermis studied. After 15 days the central band is no higher than the regions to either side and the average overall frequency is 0.02 mitotic figures per 100 μm, which lies at the lower end of the range for unoperated legs.

Experimental grafts

The early behaviour of cells in the vicinity of an experimental graft is similar to that of a control graft. Epidermal cells migrate around the cuticle of the graft to restore epidermal continuity. The epidermis in this region then starts to separate from the cuticle, and cells in the region of the graft/host junction become activated and start to divide. After about 10 days the cellular behaviour differs from that of a control graft. The cells of the epidermis of the tibia become larger and more clearly activated from a level just distal to the graft/host junction to about 800 μm proximal to it and, in the legs sectioned, usually more noticeable on the internal face. By 15 days more mitotic figures appear in this region and folds develop in the epidermis as it starts to form outgrowths (Fig. 7). Where outgrowths were found at this stage, one was seen in sections of the anterior/internal region (with respect to the host) and the other in sections of the posterior/internal region. Cell division continues throughout the epidermis of the outgrowths as they grow into the space between the epidermis and the cuticle (Fig. 8). They continue to grow and start to segment into tarsomeres as they develop into supernumerary tarsi.

The timing of the different stages of supernumerary formation is variable. No outgrowths are seen in the legs 10 days after grafting. At 15 days all stages of outgrowth are seen, from early epidermal folding to differentiated tarsi. At 20 days all legs show complete regenerates.

A comparison of the appearance of the epidermis (Fig. 9) with the distribution of mitotic figures (Figs 10, 11) in the graft region of five legs fixed 10 days after grafting (two sectioned vertically and three horizontally) and five legs fixed 15 days after grafting, gives the following results.

10 days after grafting, when experimental grafts look similar to control grafts, the band of high mitotic activity at the level of the graft/host junction shows a higher overall rate than for control grafts. The distribution of mitotic activity
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Fig. 4. 1.5 μm section through a colchicine-treated control leg 5 days after grafting. The epidermal cells have migrated to close the wound and epidermal continuity is established between the host and graft. hcu, host cuticle; gcu, graft cuticle; e, epidermis. Scale bar, 100 μm.

Fig. 5. 1.5 μm section through a colchicine-treated control leg 8 days after grafting. The epidermis has become activated and has separated from the cuticle proximal to the graft/host junction. Mitotic figures are visible in the epidermis (arrowed). hcu, host cuticle; gcu, graft cuticle; e, epidermis. Scale bar, 100 μm.

around the circumference is variable from one leg to another, showing little localization (10i), less activity on the internal region (10iii), more activity on the internal side (10ii and 10v), or a region of considerably higher activity on the internal/posterior region (10iv).

15 days after grafting, when the outgrowths are normally beginning to form, the distribution of mitotic activity is again quite variable.

In one case there is no sign of activation and no band of high mitotic activity (15i), another appears similar to a day-10 leg with a fairly narrow band of activation and mitotic activity which appears to be concentrated towards the
Days after grafting

Fig. 6. The proximodistal distribution of mitotic figures in legs following control grafts (based on ten sections from each of three animals on each day).

anterior and posterior faces of the leg (15ii), a third shows a wide band of moderate mitotic activity with no apparent circumferential localization (although the activation appears to be mainly on the internal face) (15iii), and two others show a wide band of activation and mitotic activity localized on the internal face of the leg (15iv and 15v), one of which is clearly forming two outgrowths (15v).

Growth of the supernumeraries

Two legs that were sectioned 15 days after grafting showed more-advanced stages of supernumerary outgrowth (e.g. Fig. 8). In both cases cell division was occurring throughout the epidermis of the outgrowth with no noticeable localization.

Types of supernumerary

Twenty-nine grafted legs were left to develop until the following ecdysis to observe the types of supernumerary that were formed.
Thirteen formed a pair of lateral tarsi, with no autonomous regeneration at the graft/host junction. These were usually situated at approximately the level at which the graft had been performed, but in one case the laterals originated from

Fig. 7. 1.5 μm section from the anterior region of a colchicine-treated experimental leg 15 days after grafting. The epidermis of the internal face of the leg on the host side of the host/graft junction has become thickened and is starting to form an outgrowth. Other sections show that a similar outgrowth is forming on the internal/posterior face of the leg. hcu, host cuticle; gcu, graft cuticle; e, epidermis; h, haemocytes; d, distal end of outgrowth. Arrows indicate mitotic figures. Scale bar, 100 μm.

Fig. 8. 1.5 μm section through a colchicine-treated experimental leg 15 days after grafting. An outgrowth is developing on the internal side of the leg, just proximal to the graft/host junction. There are mitotic figures throughout the epidermis of the outgrowth (between the large arrows). hcu, host cuticle; gcu, graft cuticle. Scale bar, 200 μm.
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the tibia–tarsus joint, a level considerably distal to the original graft level. The circumferential position of the outgrowths was varied, two originating from the anterior and posterior faces, four from the internal and seven from the external face.

Eleven formed partial autonomous regenerates, in which varying amounts of distal regeneration was seen at both the graft and host sides of the junction before the tissues fused to form the lateral outgrowths (see Fig. 1 and French, 1976b). Examination of the tarsus in these legs showed that in all cases but one it had been lost and regenerated.

The remaining five formed only small featureless bumps on the anterior and posterior faces of the tibia at the level of the graft/host junction.

Blastema formation during tarsal regeneration

To see how the regeneration of the graft tarsus (where it is lost) could be affecting the outcome of the supernumerary regeneration, the tarsus was removed from a metathoracic leg of a number of unoperated host-sized animals and the tibia was sectioned on varying days following removal. The sections showed that the regeneration blastema for the tarsus was formed between 8 and 12 days after amputation.

DISCUSSION

The formation of supernumerary legs in the cockroach can be considered in three main stages. First, wound healing restores epidermal continuity. This appears to be followed by a spread of activation and cell division into the surrounding epidermis to form a blastema. The blastema then grows by cell division throughout its epidermis to form the epidermis of the lateral supernumerary outgrowths.

Wound healing

The process of wound healing is the same in both control and experimental grafts. It involves the migration of epidermal cells over the outer surface of the

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Fig. 9. Examples drawn from vertical sections of experimental grafts 10 and 15 days after grafting. 10i. The appearance is similar to that of a control graft with little separation of the epidermis from the cuticle or epidermal folding. 10ii. There is slightly more separation from the cuticle, and more epidermal thickening on the internal side of the leg. 15i. There is considerable separation of the epidermis from the cuticle, but little folding or thickening. 15ii. There is some epidermal folding and thickening. Comparison with other sections shows that this occurs most on the posterior end of the internal face and the anterior end of the external face. 15iii. There is some epidermal folding and thickening, most of which is seen on the internal side of the leg. As in the section shown. 15iv. There is considerable epidermal thickening on the internal side, extending from 200 μm distal to 800 μm proximal to the graft/host junction. 15v. (A) (an anterior section) and (B) (a posterior section). There is considerable epidermal thickening on the internal side, extending from the graft/host junction to 800 μm proximal to it. Two outgrowths are forming, one seen in the anterior/internal epidermis (A) and the other in the posterior/internal epidermis (B). heu, host cuticle; gcu, graft cuticle; e, epidermis; sc, scab. Shading shows regions of thickened epidermis. Scale bar, 500 μm.
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graft cuticle until the host and graft cells meet and epidermal continuity is restored. It is not clear from the sections studied whether it is the graft or host epidermal cells that migrate or a combination of the two (the importance of this will be considered later). Following the restoration of continuity, cells in the vicinity of the graft/host junction become more activated, detach from the cuticle and start to divide. In *Rhodnius prolixus* cell division during wound healing is caused by depletion of epidermal cells in the area from which they have migrated (Wigglesworth, 1937), and this could also be the case in this situation.

**Formation of the blastema**

Whereas in control grafts the epidermal cells return to the resting state following wound healing, in experimental grafts the area of activated and dividing epidermal cells usually increases to form a blastema from which the supernumerary legs will develop. Where this happens the area covered by the blastema typically extends from just distal to the graft/host junction to about 800 \( \mu m \) proximal to it. The results from legs sectioned 10 and 15 days after grafting, together with those from animals left to moult, suggest that the outgrowth is not necessarily centred on the areas of maximum positional discontinuity but often occur towards the internal or external faces of the leg.

**Growth and differentiation**

As cell division continues, the blastema pushes out into the space between the epidermis and the cuticle. In one specimen, where the outgrowth was seen in its early stages (15v), the distal end of the outgrowth is about 200 \( \mu m \) proximal to the graft/host junction. This raises a problem, because in other species of cockroach and in crickets it has been shown that the cells of the graft and host epidermis normally give rise to the distal and proximal sides of the supernumeraries, respectively, with the graft-tissue/host-tissue junction extending to the distal tip of the supernumeraries (Bohn, 1972; French, 1976b; French, 1984). This could be explained in one of two ways. If cell division immediately proximal and distal to the graft/host junction produced the cells that form the blastema, the epidermis proximal to the junction would have to be displaced proximally by an amount equivalent to the length of the blastema. One would then expect to see either

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Fig. 10. Distribution of mitotic figures 10 days after grafting. 10i and 10ii. From vertical sections of legs shown in Fig. 9. (See Fig. 3 for construction of graphs.) 10i. Proximodistal graph shows a moderate rate of cell division throughout the length studied, with a higher rate at the level of the graft/host junction. The circumferential graph shows no significant localization of cell division within the band of higher mitotic activity. 10ii. Similar to 10i except that there is a rather lower background level and the band of higher mitotic activity extends slightly more in the proximal direction and has a higher level on the internal side of the leg than the external side. 10iii–10v. From horizontal sections through band of high mitotic activity. 10iii. Mitotic activity rather lower on the internal region. 10iv. A significant area of higher mitotic activity on the internal/posterior region. 10v. Mitotic activity significantly higher on the internal side than on the external side. a, anterior; p, posterior; i, internal; e, external.
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extensive epidermal folding or a higher than normal cell density, neither of which can be detected in any of the sections. Alternatively, activation could spread into the cells lying proximal to the junction to produce the blastema in a similar way to blastema formation during leg regeneration (Truby, 1983b). However, this would produce a blastema consisting largely of host cells, and for the final regenerate to consist of half graft-derived and half host-derived cells it would have to be followed by differential rates of cell division in the host- and graft-derived tissues of the blastema. Such differential rates of division are not seen in the patterns of cell division. This problem could be overcome if cell migration at the start of wound healing resulted in the location of the junction between the graft and host tissues within the blastema lying proximal to the position of the graft/host junction indicated by the proximal limit of the graft cuticle. There is nothing to suggest that this does not happen, but the graft cells would need to be labelled to show their final position in the blastema to confirm the suggestion. Attempts at labelling the host and graft cells to follow them during blastema formation (by using different species, by \[^{3}H\]thymidine labelling of the graft tissues or by incorporating a fluorescent label into the graft cells) have as yet not produced a useful marker. To follow the cell movements clearly it may be necessary to develop a species-specific immunofluorescent marker.

Another problem in distinguishing between these different explanations is the rapid rate of cell division that occurs in both control and experimental grafts following wound closure. This obscures any early patterns of cell division that may be due to pattern discontinuities and which may be involved in forming the blastema.

Blastema formation in relation to different types of supernumerary

Not all the results conform to the behaviour so far described, either in terms of the final outcome of the regeneration or in the behaviour of the epidermis and the patterns of cell division. Five grafts failed to produce full regenerates in the first moult cycle. This response could be reflected in the result seen in leg 15i where there is very limited folding or cell division. A possible reason for this could relate to the fact that there is a critical point in the moult cycle of the cockroach after which blastema formation cannot occur but blastema growth can (O’Farrell &

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Fig. 11. Distribution of mitotic figures 15 days after grafting. From vertical sections of legs shown in Fig. 9 (see Fig. 3 for construction of graphs). 15i. This sample shows a moderate level of cell division throughout, with only a slight increase at the graft/host level. 15ii. This shows a much higher rate of cell division in the region of the graft/host junction. Within this region there is most cell division towards the posterior end of the internal face and the anterior end of the external face. 15iii. Here the rate of cell division in the central band is not so great but extends 700 μm proximal to the junction on both sides of the leg. 15iv. The central band of higher mitotic activity is only present on the internal side, but it extends 800 μm proximal to the junction. Around the internal side of the leg there is no further localization of mitotic activity. 15v. Similar to 15iv except that the band does not extend so far from the junction. The arrows mark the positions of the distal ends of the outgrowths shown in Fig. 9.
Stock, 1953; Truby, 1985). If the blastema that will form the supernumeraries has not formed before this point, supernumerary regeneration may be delayed until the following moult cycle.

Another class of results were the semi-autonomous regenerates. It has been suggested that these could result from a failure of the wound to heal before the two cut ends have undergone some independent terminal regeneration (French, 1976b). An alternative explanation in terms of blastema formation could be that a rather larger than usual blastema is formed, extending around the whole circumference of the leg, such as is seen in leg 15iii. This could be due to the amount of activation and cell division that is required for wound healing. When the cells of such a blastema become determined there is no reason why the new pattern should not be that of a semi- or even fully autonomous regenerate. This would explain the fact that grafts with no reversal of axes have also been known to produce varying degrees of autonomous regeneration (French, 1976a). It could also account for the fact that the formation of a tarsal regeneration blastema increases the likelihood of autonomous regenerates being formed, or in some cases of the base of the supernumeraries being at a more distal level than that at which the graft was originally performed. The tarsal regeneration blastema would be present during the activation and early cell division stage following the graft, and as it appears to extend back to the graft level it could cause the production of a larger than normal area of blastema. Also, the cells of the tarsal regeneration blastema all end up forming more-distal levels of the leg than that from which they were derived. If the tarsal blastema overlapped the supernumerary blastema the supernumeraries would also have their bases at a more distal level.

CONCLUSION

During the formation of supernumerary outgrowths, the new epidermis is produced by cell division with a blastema in the region of the graft/host junction, where normally non-adjacent parts of epidermis are confronted. However, this cell division is not restricted to the tip of the blastema and there is no evidence that the blastema is formed by local cell division at the graft/host junction. It seems more likely that, as with leg regeneration following amputation, the blastema is formed by a spread of cell activation into the surrounding epidermis. The blastema then grows by cell division throughout its epidermis to form the regenerated structure (Truby, 1983a,b).

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REFERENCES

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