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REACTIONS TO LEG EXCISION IN LARVAE OF *ENALLAGMA CYATHIGERUM* (CHARPENTIER) (ZYGOPTERA: COENAGRIONIDAE)

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Soon after amputation of a leg from a final instar larva, granular haemocytes, supported by strands of clotted haemolymph plasma, form a thin cap over the wound, providing the initial wound closure. Cut nerves also appear to attract granular haemocytes and promote organization of a cell sheet covering the wound. Gradually, congregating cells transform the original sheet of cells into a sealing haemocytic plug. Within and across the plug, granular cells form a densely-packed, primary layer like a diaphragm that soon becomes melanized. This is followed later by one or more deeper, secondary layers which also melanize, but to a lesser extent. The primary, melanized layer serves as a pseudocuticle. Its position appears to be determined by the reorganizing epidermis at the wound edge. Presumably the melanized cell layers reduce leakage of haemolymph plasma from the wound and establish barriers against intruding microbes and other foreign bodies. It is suggested that the sequence of cellular events is similar in all kinds of lesions caused to the zygopteran integument, whether by ectoparasitic invasion or physical damage. It is likely that a resultant alteration in the properties of the subepidermal basement membrane will elicit the haemocytic response.

INTRODUCTION

During the healing of wounds inflicted on the insect cuticle/epidermis, haemocyte congregation plays a principal role among the cellular events (SALT, 1970; ROWLEY & RATCLIFFE, 1981). As insect haemocytes display a variety of functions, the question arises whether the healing processes following physical injuries differ from such reactions as, for instance, haemocytic defence against penetration of the insect cuticle by invading parasites.

Zygopteran adults have their cuticle pierced by the mouthparts of ectoparasitic water-mite larvae; subsequently, within the host tissues, each larval mite produces a gelatinous, resilient feeding tubule, the stylostome, which eventually elicits a

sequence of haemocytic responses terminating in encapsulation (ÅBRO, 1979, 1982, 1984, 1992). The present study was initiated to elucidate whether reactive alterations at the bite site of water-mite larvae, caused by the influx of foreign organic substances, comprise specific defence reactions against the parasites, or if these cellular events reflect a general pattern of wound repair. To avoid complications because of instantaneous coagulation reaction, or desiccation and formation of crusts in the wound on exposure of haemolymph to the air, the early stages of wound repair have been surveyed in final instar zygopteran larvae.

MATERIAL AND METHODS

Final instar larvae of the zygopteran *Enallagma cyathigerum* awaiting metamorphosis, were collected during early spring near Bergen, western Norway, and transferred to a laboratory to be reared at a water temperature of 12° ± 1°C and with a periodic alternation of 12 hours of artificial illumination and 12 hours of dark. One hind leg was cut off, using iridectomy scissors, under water in a wide Petri dish.

Larvae were fixed at various times after amputation, i.e. at 30 minutes, 1 hour, and at 1 and 3 days. Fixing fluid was gently injected into the larvae near the damaged leg with a fine hypodermic needle. This was carried out under a surgical operating microscope. Fixation took place at 12°C in 2.5% glutaraldehyde in a cacodylate buffer (pH 7.3) with postfixation in 1% osmium tetroxide in the same buffer. The specimens were then rinsed in the buffer alone, dehydrated in ethanol and cleared in propylene oxide. They were embedded in epoxy resin and semithin sections (1-2µm) were cut, stained with toluidine blue and viewed under a light microscope; ultrathin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate and viewed in an electron microscope.

Stains for melanin in semithin and ultrathin sections of epoxy resin were applied according to a modified Warthin-Starry procedure (VAN DUINEN et al., 1983). Minute amounts of melanin can thus be demonstrated, indicating high sensitivity.

Terms and classification of haemocytes are based on the accounts of GUPTA (1979) and BREHÉLIN & ZACHARY (1986).

RESULTS

SURVEY VIEW

Under the microscope, haemolymph plasma could be seen immediately oozing from the wound following amputation. Soon minute granules appeared accumulating on cut nerves and tracheae. After 30 minutes, a thin granular coating layer over the wound was recognizeable. Histological sections from this stage, examined by low power light microscopy, revealed a coating of a few cells' thickness spread across the hole (Fig. 1a).

At 1 hour, additional cells had assembled in the wound. A layer of tightly packed cells appeared inside the original, coating cell layer, and, near the cut level, the epidermis had started to loosen from the cuticle or had disappeared entirely (Fig. 1b).

At 1 day, a closing cell plug exhibiting stratification had established itself in the

wound, with a thick layer of coagulated haemolymph plasma and ruptured cells on the outside. Inside this, across the wound, there was a distinct, tight opaque layer that stained heavily for the presence of melanin. Innermost was a rather thick, cellular mass about to organize against the haemocoel a secondary, opaque layer that also stained positively for melanin. At this stage, the epidermal layer had de-

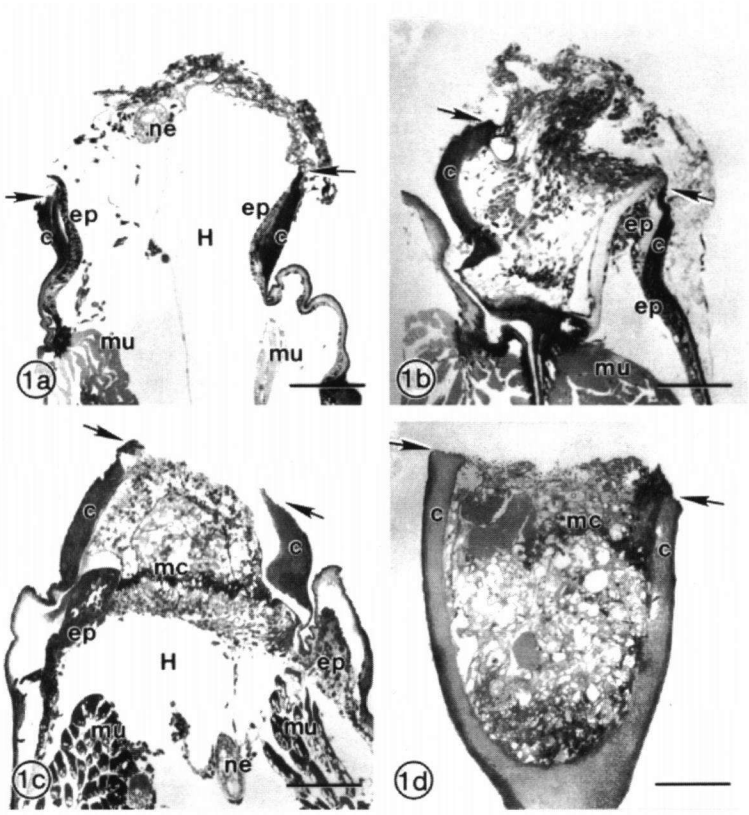


Fig. 1. Representative light micrographs (longitudinal sections) through the healing stump after amputation of the third leg in a final instar larva of *Enallagma cyathigerum*, demonstrating structural alterations. Arrows indicate original level of amputation. Toluidine blue. Scale bar = 100 µm. [c: cuticle; – ep: epidermis; – H: haemocoele; – mc: melanized cell layer; – mu: muscle; – ne: nerve]: (a) At 30 minutes, circulating haemocytes organize a covering cap, a few cells thick, over the wound. The epidermis (ep) is seen extending to the cut edge; – (b) At 1 hour, the wound was closed by a plug consisting mainly of a mass of clumped haemocytes and coagulated haemolymph plasma. Note loosening and disappearance of epidermis near cut edge; – (c) After 1 day, a layer of dense and dark-staining cells (mc) makes a pronounced demarcation in the plug between an outer mass consisting mostly of clotted haemolymph plasma and cell debris and an inner, distinct cellular region. Epidermal cells are absent outside the level of the dark layer. Note vacuolization of muscle fibres (mu); – (d) After 3 days, the wound plug extends considerably deeper in the stump. The dark demarcation layer (mc) now appears discontinuous.

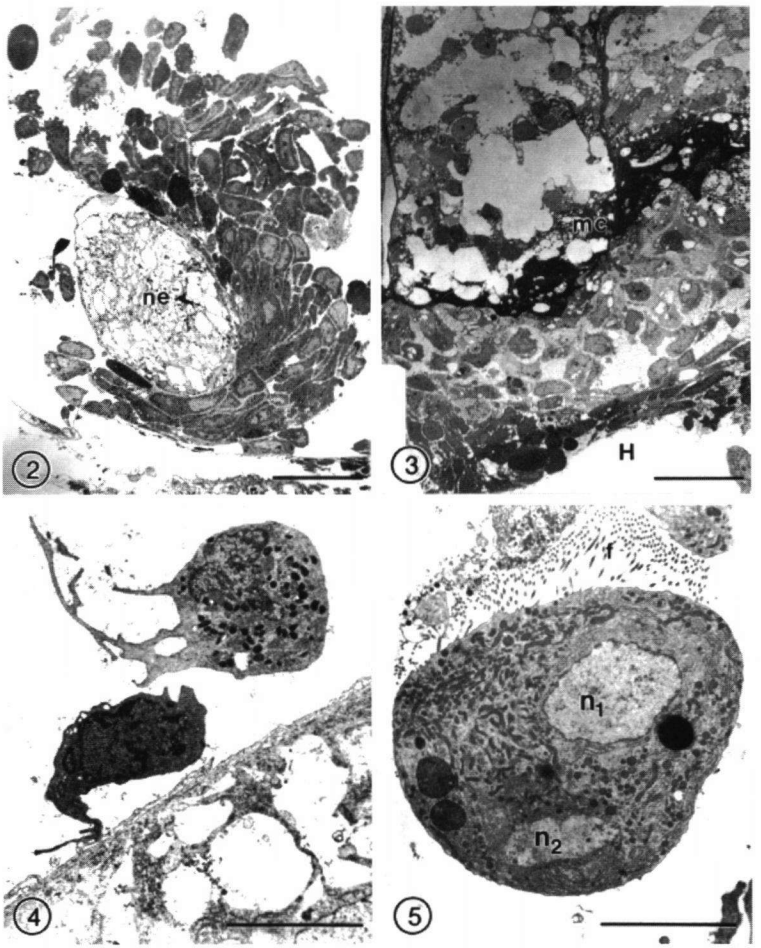
tached from the cuticle at the cut level. Where the epidermal layer maintained its adherence to the cuticle, the epidermis had formed a conspicuous thickening. The primary melanotic layer made its appearance just at the level of the thickened layer of epidermal cells (Fig. 1c).

After 3 days, the wound plug had grown deeper underneath, and the tightly--packed dense layers, the primary and secondary ones, were still melanized but discontinuous (Fig. 1d).

FINE STRUCTURE

After amputation, granular haemocytes initially aggregated round the nerve stump. At first the settled cells appeared almost unchanged, with only a little distortion of their shape. Later the cells became tightly-packed. The cells adhered to the surface of the nerve and appeared to be very cohesive (Fig. 2). While the inner cells flattened themselves upon the surface of the nerve, the haemocytes that were somewhat removed from the nerve attained a spindle shape or a rounded outline. Away from the nerve, granulocytes organized themselves in a cell sheet approaching the cut edge of the cuticle, which to some extent was supported by strands of clotted haemolymph plasma, thus forming a cap that sealed the lesion. Gradually, epidermal cells near the cut edge separated from the cuticle. Disconnected epidermal cells seemed to join the cell aggregate that was coming into being centrally in the wound. Also, the outermost cells of the accumulation were seen to become dilated, vacuolated, and later ruptured. There were no instances of melanotic incrustation of the surface of nerves. In zygopteran specimens that had had the nerve pulled out of the leg stump immediately after amputation by fine-pointed tweezers, the cell cap formation and aggregation were postponed considerably.

In the course of the first day the outer portion of the rather loose wound plug changed into a meshwork of threads, necrotic cells and debris, wherein abundant bacteria-like rods could be seen (Fig. 6). Where the epidermal layer maintained its adherence to the cuticle, the epidermis had formed a conspicuous thickening, consisting of several dividing cells. Bacteria were not found inside the electron-dense layer of granulocytes that stained heavily for melanin (Fig. 3). As the cell plasma became denser, structural modifications occurred, such as a decrease in the number of cytoplasmic granules and a temporary rise of intracytoplasmic microtubules. Deeper in the plug, granulocytes could sometimes be found containing endoplasmic bacteroid bodies of indwelling origin (Fig. 7), which normally are not seen in these cells. Granulocytes constituted a majority of cells in the wound plug. During the organization of the wound plug, single large cells appeared in the wound area. These could exhibit more than one nuclear profile and had developed a brush--border of filopodia on the side nearest to the damaged epidermis. These cells harboured a large number of elongated mitochondria, a pronounced granular endoplasmic reticulum, some rounded, rather dense bodies, and a few inclusion bod-

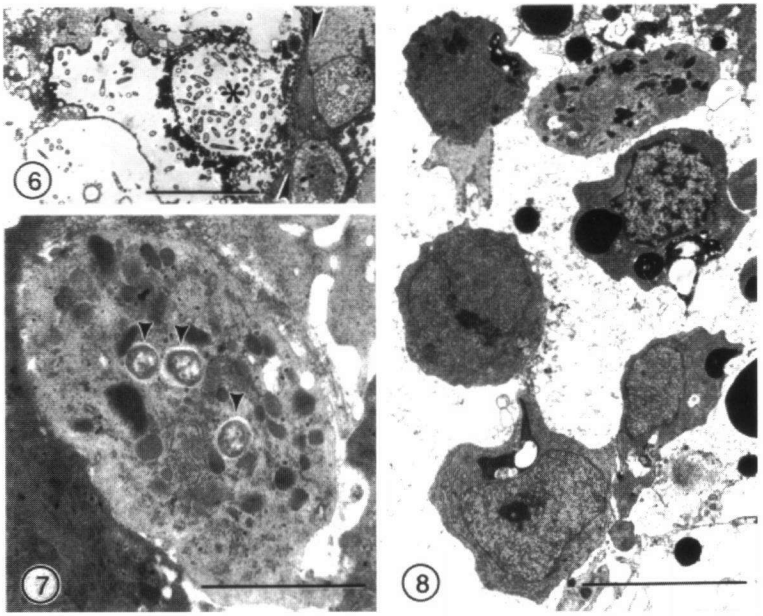


Figs 2-5. *Enallagma cyathigerum* [Scale bar = 10μm]: (2) Low power electron micrograph showing granular haemocytes assembling around a nerve stump near the cut. Settled haemocytes flatten on the nerve (ne), similar to incipient encapsulation. 30 minutes; – (3) Electron micrograph showing different layers of the wound plug. The dense transverse layer consists of tightly packed, melanized cells (mc). The upper half shows the outer portion with strands of coagulated haemolymph plasma and decomposing cells. In the lower half there is a compartment consisting of several types of cells, of which the deepest ones are organizing a secondary layer of compression and melanization. H: haemocoele. 1 day; – (4) A granulocyte with long cytoplasmic extensions and an agranular epidermoid cell with cytoplasmic projection near an accumulation of vacuolated degenerating cells. 1 day; – (5) A large free cell forming filopodia (f) near decomposing epidermal cells. Note the presence of two nuclear profiles (n_1 and n_2). 1 hour.

ies of variable substructure (Fig. 5).

During the first day several, single granulocytes with long, slender projections were found deep in the wound plug beneath the primary melanized layer. They often occurred together with single, small agranular cells, also with cytoplasmic projections (Fig. 4).

As the wound plug grew in depth into the stump after amputation and a primary melanotic cell layer had formed, one or two successive, less dense, layers appeared deeper in the plug. For the most part they seemed to be recruited from granular haemocytes but stained faintly for melanin. In portions of the plug containing decomposed cells, epidermoid cells could be found spread over the scar, forming more or less continuous cords of cells containing phagosomes and electron dense spherules of debris (Fig. 8).



Figs 6-8. *Enallagma cyathigerum*: (6) Coat of debris with abundant bacteria-like rods (asterisk) outside the outermost protective layer of tightly packed cells (arrowheads) in the wound plug. 1 day. [Scale bar = 10μm]; – (7) Endoplasmic bacteroid bodies (arrowheads) within a granulocyte in the organizing wound plug at 1 hour. [Scale bar = 5μm]; – (8) Cells with phagosomes and electron-dense bodies (spherules) from decomposed cells deep in the wound plug at 3 days. The spherules exhibit an internal substructure. Note that the shape of these cells is influenced by large and numerous inclusions. [Scale bar = 10μm].

DISCUSSION

The cellular healing process of the insect integument after experimental damage has been studied by several workers (e.g. GÖTZ, 1986). It can be subdivided into (1) migration of haemocytes to the wound site, (2) involvement of granular haemocytes in an immediate clotting reaction of the haemolymph plasma, and (3) organization of a cellular wound plug. Accumulation of granular haemocytes and clotting of the haemolymph plasma appear to be common features of wound repair in insects of all ages (SALT, 1970; GÖTZ, 1986). As with insects generally, it is presumed that the healing process in Zygoptera follows similar courses in both larvae and adults. In the zygopteran larvae studied, granular haemocyte (= granulocyte) behaviour corresponds in several ways to that of granulocytes observed during wound repair in locusts (BREHÉLIN et al., 1976) and is consistent with the general method of regeneration in insects.

In the present excision experiments, the healing process was not surveyed long enough to be able to identify the re-establishment of a continuous epidermis/cuticle. When interpreting the observations it should be emphasized that the wounding took place under water. Exposed larval cells in the wound may imbibe water, thus bringing about a vacuolation of cytoplasm and a subsequent cell rupture. In the present study, granulocytes play a leading role, first by forming the protective cap, a sort of pseudo-epithelium that covers the wound, and subsequently by promoting the more enduring closure by composing and organizing a sealing plug of clumped cells. The formation of the cell cap over the wound, together with the clotting of the haemolymph plasma, helps to reduce and finally to stop bleeding. The coagulum is hardened, presumably by the release of activating agents from the granulocytes (BOHN, 1986).

Granulocytes participating in the emergence of diaphragms of tightly packed melanotic cells within and across the haemocytic plug comprised an important feature of wound repair in the zygopterans. The formation of such blocking barriers seems to constitute a most efficient closure for the prevention of both haemolymph leakage and the invasion/influx of microbes or other foreign substances. Insects possess a phenoloxidase system for hardening and tanning their cuticle (GÖTZ, 1986) and this enzyme system participates also in wound healing and defence reactions against parasites (YOSHINO & VASTA, 1996).

In the wound, epidermal cells heaped up along the margin of the remaining epidermis layer appear to exercise an organizing influence on the emergence of the primary, melanotic cell layer contributed by granulocytes. Large cells exhibiting a brush-border of filopodia are likely to be multinucleate and may be derived from epidermal cells in the wound area simply by fusion; this could not be stated definitely, as also other types of insect cells can be induced to form filopodia (LOCKE, 1987).

In addition to the damaged integument, injured nerves seem to release substances

that attract circulating haemocytes. This is similar to the migration of granulocytes towards foreign bodies of organic material inserted into the haemocoele of an insect. Also they behave in much the same way as when they settle on foreign bodies to commence encapsulation, the innermost cells transforming into flattening lamellocytes (ÅBRO, 1979, 1982), but yet without trace of melanization. Furthermore, a cut nerve in the wound appears to promote the development of a covering wound cap and the organization of a subsequent plug.

The early, rapid clotting, important in wound sealing, is achieved by the disintegration of fragile haemocytes, which provides coagulation proteins to carry out immediate gelation. The haemocyte coagulogen builds up the basic structure of the clot, which subsequently becomes stabilized by the plasma coagulogen (BOHN, 1986). The primary melanotic barrier constitutes a distinct demarcation in the plug between an outer mass, consisting of clotted haemolymph plasma and cellular debris, and an inner zone of rather undamaged cellular structures. This primary melanotic layer constitutes a pseudo-cuticle beneath which the congregated cells perform as a pseudo-epithelium.

Bacteroid bodies seen in the granulocytes resemble rickettsia, as judged by morphological features alone. This appears to be a kind of bacterial endocytobiosis or endosymbiosis, and has been observed in other zygopteran cells (Åbro, unpublished).

Puncture of the zygopteran host cuticle by ectoparasitic water-mite larvae (*Arrenurus* spp.) and the successive development within the host's epidermis layer of a feeding device, the stylostome, will eventually cause a rupture of the subepidermal basal lamina, as the stylostome enters the haemocoele to become exposed to circulating haemocytes. Granulocytes may aggregate close to the stylostome to initiate its encapsulation (ÅBRO, 1979, 1982, 1984). Based on the present experiments, this cellular behaviour does not appear peculiar to parasitic influence but rather reflects a normal pattern which applies to every type of lesion of the zygopteran integument. It appears that any local injury to the subepidermal basal lamina or basement membrane will trigger a cellular inrush of haemocytes to the site.

The two main cellular defence reactions in insects are phagocytosis and encapsulation, and a depression of these reactions could be brought about by parasites or pathogens. A combination of haemocytic and humoral reactions contribute to the encapsulation of foreign material (YOSHINO & VASTA, 1996). The similarities of melanotic encapsulation of parasitic material and, within the wound plug, the development of melanotic barriers against agents from the outside, indicate two variants of the same fundamental defence process.

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