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A BRIEF REVIEW OF THE IMMUNOBIOLOGY OF *ELEDONE CIRRHOSA*

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Blood can be sampled repeatedly from the lesser octopus *Eledone cirrhosa* (Lamarck) and the haemocytes cultured for up to 72 h. Sampling results in an increase in the number of circulating haemocytes per ml and in the percentage of haemocytes containing cytoplasmic granules, and a change in the staining pattern of the haemocytes. It also causes a decrease in the quantity of copper in the haemolymph. The haemocytes phagocytose bacteria (*Vibrio anguillarum*) *in vitro* in the absence of haemolymph, but there is enhanced phagocytosis in the presence of haemolymph. Haemocytes migrate towards low concentrations of various blood preparations, to lipopolysaccharide (LPS) and to preparations that had contained live bacteria. They also have an apparent bacteriostatic effect on the growth of live bacteria. Haemocytes produce intracellular reactive oxygen species after incubation with dead bacteria in particular, but also live bacteria and LPS. *E. cirrhosa* haemolymph agglutinates the bacteria *V. anguillarum, V. parahaemolyticus* and *Aeromonas salmonicida* and exerts a bacteriostatic effect on them. The haemolymph, haemocytes and certain tissues from *E. cirrhosa* exhibit lysozyme and antiprotease activity. Injection of live *V. anguillarum* causes an increase in lysozyme activity in the branchial heart (after 48 h) and a decrease in the number of circulating haemocytes (after 24 h). Antiprotease activity increases in the haemocytes (4 h) after injection of bacteria but decreases in the haemolymph. Live bacteria cause an increase in the number of circulating haemocytes, but are cleared from the circulation in about 4 h by both the haemocytes and tissues (branchial heart, branchial heart appendage and white body), where they are degraded. Ultrastructural changes were observed in the branchial heart cells and the haemocyte nucleus after injection of bacteria. Following injection, colloidal graphite is aggregated in blood vessels only.

Immune systems in animals involve complex combinations of humoral and cellular components, as well as external barriers, that act to prevent invasion by pathogens. Although invertebrates lack the highly specific immune factors, such as immunoglobulins (Igs) and other variable region molecules (Marchalonis and Schluter 1990, Cooper *et al*. 1992), that are characteristic of the vertebrate immune response (Manning and Turner 1976), the invertebrate immune system is adequate for the survival of each species (Klein 1989). External barriers, such as mucus, various arrangements of epithelial cells, and exoskeletons together with antimicrobial and modulating factors, serve to prevent external entry by pathogens (Ratcliffe *et al*. 1985, Millar and Ratcliffe 1994). If the external barriers are breached, the internal cellular and humoral components of the invertebrate immune system act together to destroy the invader. Cellular defence activities performed by invertebrate blood cells include wound repair, coagulation, phagocytosis, encapsulation, nodule formation, and the production of various antimicrobial and cytotoxic substances (Ratcliffe *et al*. 1985, Pagliara *et al*. 1993, Millar and Ratcliffe 1994). Invertebrate humoral immunity is characterized by factors present in the plasma or serum, such as agglutinins and various antimicrobial and immune modulating components, which could have originated from the blood cells or other tissues and which are involved in various defence activities (Ratcliffe *et al*. 1985, Smith and Chisholm 1992, Millar and Ratcliffe 1994).

MOLLUSCAN IMMUNOBIOLOGY

Molluscs share with other invertebrates a number of similar immune functions, such as phagocytosis and agglutination. They are a diverse animal group and include gastropods, bivalves and cephalopods. However, until recently, most of the studies concerned with their immunobiology have concentrated on gastropods and bivalves only (Bayne 1983).

Cephalopod immunobiology

Cephalopods are soft-bodied advanced molluscs that inhabit a variety of marine environments. Various

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Table I: Humoral and cellular immune reactions found in *E. cirrhosa*

Response	Organs	Type of response
Cellular	Haemocyte	Numbers per ml increase after sampling Numbers per ml with cytoplasmic granules increase after sampling Phagocytosis/enhanced phagocytosis Migration Bacteriostatic activity Intracellular reactive oxygen species production Lysozyme activity Antiprotease activity
	Branchial heart	Lysozyme activity after bacterial injection
	Branchial heart, branchial heart appendage and the white body	Involved in *in vivo* bacterial clearance
Humoral	Haemolymph	Sampling causes a decrease in total copper Opsonization Agglutination Bacteriostatic activity Lysozyme activity Antiprotease activity

studies using captive cephalopods have demonstrated that, once injured, their wounds are invaded by various opportunistic bacteria; this infection can be fatal (Leibovitz *et al*. 1977, Hanlon *et al*. 1984, Ford *et al*. 1986, Bullock *et al*. 1987, Hanlon and Forsythe 1990, Ford 1992). In culture conditions, various other factors, such as temperature and condition of the seawater, may also have a detrimental effect on their survival (Reimschuessel and Stoskopk 1990).

Cephalopods have a closed circulatory system encompassing a central systemic and two branchial (gill) hearts (Wells 1978, 1983, Wells and Smith 1987). The blood is pumped through elastic arteries, veins and "capillary beds", similar to the vertebrate circulatory system (Browning 1979, Wells 1983, Shadwick and Nilsson 1990); in addition, it is "stored" in large blood sinuses (O'Dor and Wells 1984). The blood consists of haemolymph (plasma) which contains dissolved protein; 98% of that protein is the respiratory pigment haemocyanin (Ghiretti 1966, Wells 1983). Only one type of haemocyte (blood cell) has been described; it originates and matures in the leucopoietic organ (white body), situated in the orbital sockets behind the eyes (Cazal and Bogoraze 1943, Cowden 1972, Cowden and Curtis 1974, 1981, Wells 1978). As with other molluscs, cephalopod blood does not clot and, when an animal is wounded, blood loss is prevented by vasoconstriction of the muscles surrounding the wound. Haemocytes aggregrate at the wound site and form a sealing plug (Polglase *et al*. 1983, Wells 1983, Féral 1988, Ford 1992).

Recent experiments have shown that the haemolymph, haemocytes and various other organs of the lesser octopus *Eledone cirrhosa* (Lamarck) exhibit cellular and humoral immune reactions (Table I) that interact to protect the octopod against potentially pathogenic bacteria if the external barriers, such as the skin and mucus, are breached (Malham 1996).

BLOOD SAMPLING

In order to study the cellular and humoral components of the immune system of *E. cirrhosa*, it was necessary to sample adequate quantities of blood (repeated samples if needed), to be able to separate the haemocytes from the haemolymph, and to culture the haemocytes *in vitro* under controlled conditions. The procedure to sample blood involves anaesthetizing the animal and partially reflexing the mantle to expose the branchial blood vessel. The blood is then withdrawn and put directly into ice-cold marine anticoagulant to prevent haemocyte clumping. The haemocytes can also be cultured for up to 72 h by removal of the marine anticoagulant and resuspension of the haemocytes in an octopus Ringer solution.

The same sampling and culture techniques (Malham *et al*. 1995) can be used in other studies of the immune system of cephalopods or in assessing their health in captivity, determination of their reproductive development, and determining the effects of pollutants.

Effects of blood sampling

In *E. cirrhosa*, sampling and repeated sampling of blood is known to affect the number of circulating haemocytes and the concentration of copper present in the haemolymph (Malham 1996). Haemocyte counts

increased within 2 h, reverted to near starting values after 4–5 days, and then increased again to the end of the sampling period (24 days). During the first four repeated sampling days, the increase in the number of haemocytes was concomitant with an increase in the percentage of haemocytes containing visible cytoplasmic granules, which reverted to near normal levels by Day 24. Acid phosphatase, diaminobenzidine, bromophenol blue and periodic acid Schiff's reaction gave variable staining results over 10 and 24 days.

The concentration of copper in the haemolymph decreased within 24 h, and further sampling between Days 1 and 3 showed no significant change. Continued sampling over the next 11 days, however, caused the copper concentration to decrease significantly. Haemolymph protein values decreased only within the first 2 h of sampling; thereafter, they showed no significant change over 7 days.

It appeared that, initially, the increase in haemocyte numbers per ml attributable to sampling was caused by loss of blood, i.e. it was a wounding response. Concomitant with the increase was a change in the staining pattern of the haemocytes and an increase in the percentage of haemocytes containing cytoplasmic granules. Further investigation is required as to whether these "new" haemocytes are released from the leucopoietic organ, or white body (SKM unpublished data), or other stores (such as the posterior salivary gland, the space around the white body, or the branchial heart) or by proliferation of circulating haemocytes. Cowden and Curtis (1974, 1981) showed that, in octopuses, haemocytes mature in the white body and, upon release, contain numerous cytoplasmic granules. Necco and Martin (1963) suggested that maturing haemocytes are held in the white body at telophase. If that is true, then large numbers of newly mature haemocytes could be released quickly if needed. It would appear then that loss of blood caused the release of haemocytes that were mature, or quickly completed maturation, and it is those cells that had the numerous cytoplasmic granules.

Interestingly, during repeat sampling, haemocyte numbers per ml and the percentage of haemocytes with cytoplasmic granules increased over 3–5 days. Thereafter, the number of haemocytes per ml decreased, and then secondarily increased again, whereas the percentage of haemocytes with cytoplasmic granules continually decreased after Day 5. Consequently, the time of sampling was very important. If newly released haemocytes contain cytoplasmic granules, then only a certain number of "new" haemocytes can be released over a particular period of time. The fact that samples from previously unsampled animals showed only a small percentage of haemocytes with cytoplasmic granules may indicate that the granules were either released by exocytosis or were used within the cell during the life cycle of the circulating haemocyte. Judging from the number of haemocytes with granules, only about 10% of the circulating haemocytes were recently released. The secondary increase in haemocytes per ml could have been due to a release of stored already-mature haemocytes or, though less likely, to a release of haemocytes that were not yet fully mature (i.e. they had not yet developed their full complement of granules), or to a proliferation of circulating haemocytes. To date, very little is known about the life cycle and replacement rate of a circulating haemocyte and studies of its activities and life span, combined with studies of the white body, are needed.

For aerobic animals, the decrease in the quantity of copper in the haemolymph attributable to sampling is extremely serious, because it could lead to a fatal decrease in the oxygen-carrying capacity of the blood. Although protein levels decrease over a short sampling period, they do return to more or less original values over extended sampling times. Therefore, although the protein concentration is restored, it is not in the form of the respiratory protein haemocyanin. Whether the replaced protein is in the form of apohaemocyanin remains to be seen. Interestingly, when sampled for three consecutive days (2, 3 and 4) over an 11-day sampling period, the copper content of the haemolymph remained stable for those three days only and continued to decrease from Day 5 to Day 11. Preliminary data suggest that sampling once every 5–6 days allows the quantity of copper to return to its original value. This effect, however, needs urgent further investigation, in conjunction with measurements of the blood volume.

CELLULAR DEFENCE

Similar to the situation in other invertebrates, haemocytes from *E. cirrhosa* phagocytose bacteria (Malham *et al*. 1997). Phagocytosis occurs *in vitro* in the absence of haemolymph and is affected by temperature and the duration of haemocyte incubation with formalised bacteria (*Vibrio anguillarum*). Phagocytosis increases if the bacteria are pre-incubated in haemolymph (10–100% concentrations), suggesting that opsonins are present. Stuart (1968) showed that erythrocytes were only phagocytosed by *E. cirrhosa* haemocytes after pre-incubation in haemolymph; therefore, the opsonin (soluble humoral factor(s)) seems to act as a recognition molecule (lectin), or carbohydrate binding protein, on the surface of particles either to initiate or to enhance phagocytosis. This in turn indicates that *in vivo* invading bacteria are first opsonized and then quickly phagocytosed.

Opsonization and phagocytosis of bacteria are affected by a number of parameters. In particular, when bacteria are only opsonized for a short time (1–10 minutes) at

low temperatures (5–10°C), the phagocytic rate decreases below that obtained for unopsonized bacteria. As suggested by Fryer and Bayne (1989), initial non-specific adsorption of a variety of plasma components (including opsonins) onto the surface of the bacterium could explain this inhibition. Longer exposure of the bacteria to haemolymph at higher temperatures seems to allow more of the opsonin to bind and, therefore, permits faster recognition and increased phagocytosis. Other factors that may affect enhanced phagocytosis by *E. cirrhosa* haemocytes include the presence of the divalent ions calcium and magnesium and the chelating agents ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-bis (ß-aminoethyl ether) tetraacetic acid (EGTA) that are used to dilute the haemolymph. This indicates that the attachment of the opsonin to the bacterium depends on the presence of divalent cations. However, further investigations into the nature of the opsonin(s) are necessary, and it would be interesting to know the effects of the surface properties of particles, their size and any bound ions, on opsonization and subsequent phagocytosis by haemocytes.

Haemocytes migrate under certain conditions (Malham 1996). The fact that they migrate towards low concentrations of various blood preparations (haemolymph, haemocyte lysate, whole blood and whole blood lysate) again indicates a response to wounding. Molluscan blood does not clot, but in cephalopods, haemocytes will migrate into a wound and seal it (Polglase *et al.* 1983, Wells 1983, Féral 1988, Ford 1992). It would seem therefore, that *in vivo* haemocytes would move towards an area of diluted blood (seawater may leak into a wound), which would probably also contain lysed haemocytes. These migrating haemocytes would then plug the wound and phagocytose any necrotic tissue. Haemocytes also move towards blood preparations that have been incubated with live bacteria, suggesting that some of the bacterial secretions are chemoattractant(s). *In vivo*, wounding can also lead to an invasion of live bacteria which, upon release of certain substances, attract the haemocytes. Haemocytes *in vitro* migrate towards blood preparations that contain lipopolysaccharide (LPS, Malham 1996). As LPS is part of the cell wall of bacteria, haemocytes may not only recognize secreted bacterial products (from live bacteria), but they will also recognize constituent molecules from bacterial walls.

Interestingly, there was negative migration when blood preparations (haemolymph in particular) were used in which dead bacteria had been incubated. However, phagocytosis of dead bacteria did occur (Malham *et al.* 1997), although more information is needed on the nature and effects of the chemo-attractants that are secreted by live bacteria and whether other molecules, such as other bacterial constituents, also induce haemocyte migration. Furthermore, from the phylogenetic point of view, it would be interesting to know whether molecules that induce vertebrate macrophage migration, such as tumour necrosis factors and interleukins, also stimulate *E. cirrhosa* haemocytes.

E. cirrhosa haemocytes are capable of inhibiting the growth of the bacteria *Vibrio anguillarum, V. parahaemolyticus* and *Aeromonas salmonicida in vitro* (Malham 1996). However, this inhibition varied with the type and concentration of bacteria used, the incubation temperature (5–20°C), and the duration of the experiment (0, 3 and 6 h).

Electron microscope data suggest that haemocytes digest bacteria once internalized (Malham 1996, Malham *et al.* 1997). The decrease in the number of live bacteria after incubation with haemocytes from *E. cirrhosa* is likely to be due to phagocytosis (Malham 1996). Although it cannot be excluded, it is unlikely that the haemocytes secrete bacteriostatic molecules, because nitroblue tetrazolium (NBT) reduction experiments with *E. cirrhosa* showed that both live and dead bacteria and, intriguingly, LPS, caused intracellular reactive oxygen intermediate (ROI) production. This, in turn, indicates that phagocytosis, or possibly pinocytosis with LPS, has taken place. In addition, haemocytes contain lysosomal enzymes, such as acid phosphatase, and show lysozyme and antiprotease activity (Malham 1996, Malham *et al.* 1998). These could protect against bacterial proteases. Presumably, other bactericidal molecules are present as well, and it would be valuable to isolate and characterize them as well as determining the exact nature of the action by haemocytes on the bacteria.

Preliminary investigations of phorbol myristate acetate (PMA, a membrane stimulator), bacteria and LPS incubated with *E. cirrhosa* haemocytes were carried out using ferricytochrome C, which detects extracellular ROI production (SKM unpublished data). However, no extracellular ROI production was seen, possibly because of the EGTA-containing buffers that were used to isolate and culture the haemocytes for the assays. Alternatively, haemocytes may not produce extracellular ROIs. Therefore, additional experiments are necessary to determine whether or not ROIs are produced extracellularly.

HUMORAL DEFENCE

Haemocyte-free haemolymph from *E. cirrhosa* is able to inhibit the growth of the bacteria *Vibrio anguillarum, V. parahaemolyticus* and *Aeromonas salmonicida* (Malham 1996). At high concentrations

of bacteria (1×10^9 cells·ml^{-1}), this growth is less inhibited at 15 and 20°C with *V. anguillarum* and at 20°C with *V. parahaemolyticus*.

V. anguillarum, V. parahaemolyticus and *A. salmonicida* are also agglutinated by the haemocyte-free haemolymph from *E. cirrhosa*. Agglutination activity is generally not affected by temperatures between 5 and 20°C. *A. salmonicida* showed significantly lower agglutination values ($\log_2$ titre) than either *V. anguillarum* or *V. parahaemolyticus* at all temperatures.

Stuart (1968) demonstrated the presence of agglutinins in *E. cirrhosa*, in addition to observing the presence of opsonins (see above), and suggested that the respiratory pigment haemocyanin was the opsonin. It would be interesting, therefore, to remove haemocyanin from the haemolymph and determine the effects on opsonization. Lectins have been demonstrated in *Octopus vulgaris* (Rögener *et al*. 1985, 1986, 1987), a close relative of *E. cirrhosa*. It is possible, therefore, that invading particles are quickly cross-linked in suspension (agglutination) and opsonized for rapid phagocytosis by the haemocytes. In addition, the haemolymph of *E. cirrhosa* has either a bacteriostatic or a bactericidal capacity (Malham 1996), as well as lysozyme and antiprotease activities (Malham *et al*. 1998). However, it remains to be seen whether the effect of the haemolymph on bacterial growth is due to bacteriostatic or bactericidal activity and what the causative agent is. It would also be interesting to know the effect of removal of haemocyanin on this activity of the haemolymph.

IN VIVO CLEARANCE

In *E. cirrhosa*, antiprotease and lysozyme activities were detected in various tissue samples as well as in the haemocytes and haemolymph (Malham *et al*. 1998). Injection of live *Vibrio anguillarum* caused an increase in lysozyme activity in the branchial heart over 48 h and a decrease in the lysozyme activity of haemocytes over 24 h. The haemocytes from animals injected with control phosphate-buffered saline (PBS) showed increased lysozyme levels within 4 h of injection, whereas it decreased after the injection of live bacteria in PBS. Neither PBS alone nor PBS with bacteria affected the lysozyme activity of the haemolymph. Bacterial injections had no effect on the antiprotease activity of the tissue samples, but they increased the antiprotease activity of the haemocytes as compared to the activity of the control haemocytes in the 4 h sample. Following bacterial injection, haemolymph antiprotease activity decreased at a greater rate than in control PBS-injected animals.

Haemocyte numbers per ml increased in both the control and the bacterial-injected animals, with a greater increase in the latter in the 4 h sample. Concomitant with the increase in the number of circulating haemocytes, live *V. anguillarum* were cleared from circulation within 4 h.

The increase in lysozyme activity in the branchial heart and decrease in the haemocytes suggests that lysozyme is produced in the branchial heart and then either utilized or released by the haemocytes. The lysozyme production may be associated with changes in the morphology of the large vacuole of the pore cells four hours after bacterial injection (Malham 1996). The lysozyme activity in the haemolymph did not change after bacterial injection (Malham *et al*. 1998).

The decrease of antiprotease activity in the haemolymph suggests that it is utilized, possibly to protect the tissues against bacterial proteases. As the blood was sampled from the animals before the injection of bacteria, it is difficult to determine whether the increase in haemocyte numbers per ml is due to blood loss or the injection, or both. However, the fact that more haemocytes per ml were detected in animals injected with bacteria than in the controls suggests that the bacteria had a greater effect than sampling alone.

E. cirrhosa was challenged with live *Vibrio anguillarum* for 4 h and 48 h and with colloidal graphite for 4 h (Malham 1996). The fixed tissues (branchial gill, branchial heart, branchial heart appendage, white body and haemocyte pellets) were examined at histological and ultrastructural levels to determine which tissues were involved in the clearance of the injected particles. The tissues were also compared to control tissues to identify any major morphological changes resulting from the bacterial or graphite challenge.

Four hours after the injection of the graphite, colloidal graphite was found aggregated in blood vessels (cf. Stuart 1968) and caused a morphological change in the nuclei of some circulating haemocytes. Evidence of bacteria in the circulating haemocytes was seen in all tissue samples. A full 48 h after the bacterial challenge, the nuclei of some haemocytes showed morphological changes and bacteria were seen in the cells of the branchial heart, branchial heart appendage and white body.

As with other molluscs, injected bacteria were cleared by circulating haemocytes and also by the branchial heart, the white body and the branchial heart appendages (Malham 1996). Bacteria undergoing degradation were seen in these tissues and in the circulating haemocytes; this confirms that potentially pathogenic bacteria can be removed from, and destroyed by, an internal cellular defence mechanism. This

mechanism, however, needs further investigation, e.g. by labelling of the bacteria with ^{14}C and fluorescein isothiocyanate (FITC). Also, the change in the large vacuole of the branchial heart cells and the changes in morphology of the haemocyte nucleus need further attention.

CONCLUSION

E. cirrhosa is able to defend itself against invading potentially pathogenic bacteria by using a combination of both the humoral and cellular mechanisms of its immune system. Wounding, or bacterial invasion, causes an increase in circulating haemocytes, followed by agglutination, opsonization and possible killing of the bacteria. The haemocytes migrate towards, phagocytose and probably kill an invader. If the invader escapes the haemocytes, other tissues are also capable of removing and killing it.

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