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Induction of degranulation and lysis of haemocytes in the freshwater crayfish, *Astacus astacus* by components of the prophenoloxidase activating system in vitro

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Summary. To study the role of the prophenoloxidase activating system, an enzyme cascade located in the haemocytes of crustaceans, in the cellular defences of the freshwater crayfish, *Astacus astacus* in vitro, monolayer cultures of mixed or separated haemocyte populations, isolated by density gradient centrifugation, were challenged with the bacterium, *Moraxella* sp. pre-coated with phenoloxidase and the other attaching proteins in crayfish haemocyte lysate (HLS), or in the case of controls, with saline or *Moraxella* sp. pre-incubated in saline alone. Examination of the coverslips 1 h after inoculation revealed that, in the mixed haemocyte cultures, most of the cells had undergone profound degranulation and lysis following exposure to the HLS-coated bacteria. Cell lysis was also evident in the experimental semigranular cell monolayers, but not in the controls, although in those controls treated with the saline-incubated bacteria, the semigranular haemocytes had undergone degranulation without lysis. In contrast, the granular cells appeared to be unaffected by the saline-incubated *Moraxella* sp., and with the HLS-coated bacteria displayed only marked degranulation. Greater numbers of bacteria were always associated with the cells or cell remnants in the experimental cultures compared to the controls. We suggest that the attaching proteins of the prophenoloxidase cascade are strong non-self signals for the haemocytes, causing them to degranulate and release previously cell-bound recognition factors into the haemolymph, where they are free to trigger activation of adjacent haemocytes.

Key words: Prophenoloxidase – Haemocytes – Degranulation – Phagocytosis – Opsonins

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Arthropods possess distinct populations of circulating haemocytes, capable of recognizing and responding to foreign materials without the aid of specific immunoglobulin. Foremost amongst the cellular activities are phagocytosis, encapsulation and nodule formation (see review by Ratcliffe and Rowley 1979). In attempts to understand the mechanism(s) initiating and controlling these phenomena, much interest has been generated in detecting the presence of opsonins or recognition factors in arthropod haemolymph. Some species appear to possess such opsonic factors while others do not (for review see Tyson et al. 1974; Chorney and Cheng 1980; Ratcliffe 1981), and where opsonin-like factors have been recorded, little is known about their biochemical nature or mode of action.

Recently, a possible recognition pathway based on the prophenoloxidase (proPO) activating system has been proposed for arthropods (Söderhäll 1982). In crustaceans, this system is located in the haemocytes and is specifically activated by non-self molecules such as β1,3-glucans from fungal cell walls (Unestam and Söderhäll 1977; Söderhäll and Unestam 1979), or lipopolysaccharides from Gram negative bacteria (Söderhäll and Häll, unpublished). The activating pathway is complex, comprises an enzyme cascade with at least one serine protease, and causes phenoloxidase together with four other proteins to become "sticky" (Söderhäll et al. 1979; Söderhäll 1982, 1983; Söderhäll et al. 1983). Evidence that the cascade plays a role in recognition of non-self has been provided by Söderhäll et al. (1983), who showed that crayfish exhibit stronger encapsulation reactions in vivo to fungal spores coated in crayfish haemocyte lysate than to fungal spores coated in plasma or buffer, and by Smith and Söderhäll (1983) who obtained significantly higher rates of phagocytosis of bacteria in vitro by treatment of *A. astacus* or crab, *Carcinus maenas*, haemocytes with β1,3-glucans. However, whilst we suggested that these responses might be mediated through the sticky proteins of the activating cascade after release from the haemocytes, data on the nature of the opsonins in crustacean haemocytes are still lacking. The present investigation was therefore carried out to compare the reactions of *A. astacus* haemocytes in vitro to a bacterium previously coated with the attaching components in crayfish haemocyte lysate with those towards the bacterium pre-incubated in saline alone. In addition, we have now developed a method for separating the various haemocyte populations of crustaceans by density gradient centrifugation (Söderhäll and Smith 1983), and further analyses were made with isolated haemocyte populations to determine the functional characteristics of each cell type and to evaluate their contribution to the overall cellular reactions in vitro.

Materials and methods

Animals

Freshwater crayfish, *Astacus astacus*, were collected and kept as described in Söderhäll and Unestam (1979). Only healthy intermolt male animals were used for experimental purposes.

Preparation of haemocyte lysate supernatant

Crayfish haemocyte lysate supernatant (HLS) was prepared according to the method of Söderhäll (1981), except that the final cell pellet was homogenized in crayfish saline (CFS), a balanced salt solution made up to resemble the ionic composition of *A. astacus* haemolymph (Smith and Söderhäll 1983), before centrifugation at 70000 g for 20 min (4° C). Phenoloxidase activity in the resulting HLS was estimated after activation with β1,3-glucans (Söderhäll and Smith 1983), and the phenoloxidase activity was ~800 units/ml/min.

Bacteria

The Gram negative bacterium, *Moraxella* sp. (NCMB 308), was grown and harvested as described in Smith and Ratcliffe (1978). Before use, the bacteria were heat-killed at 100° C (10 min), washed twice in CFS and then incubated in activated HLS ($\sim 5 \times 10^7$ ml^{-1}) for 2 h at 20° C. The HLS-coated bacteria were then thoroughly washed in CFS and finally resuspended in fresh CFS at a concentration of 10^7 ml^{-1}. To ensure that the bacteria remained as single particles, the suspension was vigorously pipetted several times to disrupt any small aggregates of the *Moraxella* sp. before addition to the haemocyte cultures. Control bacteria were similarly treated, but CFS was substituted for HLS in the opsonization step.

Preparation and treatment of mixed haemocyte monolayers

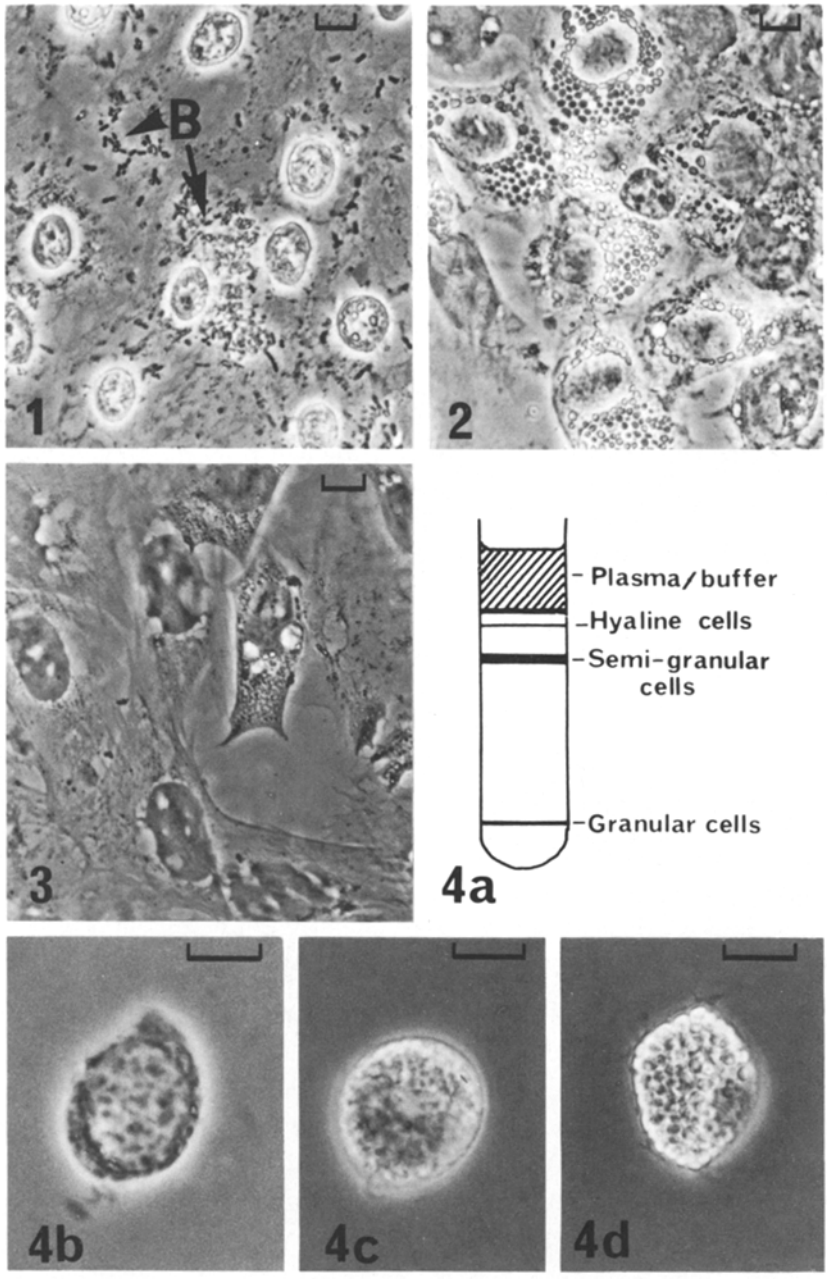
Triplicate haemocyte monolayers were prepared on clean, pyrogen-free glass coverslips from whole haemolymph as described in Smith and Söderhäll (1983). To one of the coverslips was added 100 µl of HLS-coated *Moraxella* sp., while the second received 100 µl of CFS-incubated *Moraxella* sp., and the third received 100 µl of filter sterilized CFS. The cell cultures were then incubated, rinsed, fixed and examined as previously described (Smith and Söderhäll 1983).

Separation of the different haemocyte populations

The different haemocyte population of *A. astacus* were isolated using a modification of the density gradient centrifugation technique previously reported for marine crustaceans (Söderhäll and Smith 1983). Haemolymph, 1.5 ml, was collected in 0.5 ml citrate/EDTA buffer (0.14 M NaCl; 0.1 M glucose; 30 mM trisodium citrate; 26 mM citric acid and 10 mM EDTA) pH 4.6. Then, 1.5 ml of the diluted haemolymph was centrifuged at 1.700 g for 20 min (7° C) through preformed continuous gradients of 70% Percoll (Pharmacia, Uppsala, Sweden) in 0.15 M NaCl. The resulting cell bands were removed, tested for phenoloxidase activity using the "rapid assay" described by Söderhäll and Smith (1983), and the types of haemocyte identified by phase contrast microscopy.

Preparation and treatment of the pure haemocyte monolayers

The ability of the haemocytes to attach to and spread on glass surfaces was examined by placing 100 µl cell suspension, taken directly from the gradients, together with $CaCl_2$ (20 mM final concentration) onto clean pyrogen-free glass coverslips. The cells were incubated for 20 min at 20° C, rinsed once in CFS, fixed in 10% buffered formalin, pH 7.0 and finally examined using phase-contrast optics. Haemocytes, in which the intracellular granules and/or nucleus were clearly visible were regarded as attached, while those in which the cytoplasm was also extended to form flattened cell processes were considered as spread. Phagocytic activity was measured, using freshly prepared pure haemocyte monolayers inoculated with HLS-coated or CFS-incubated *Moraxella* sp. as above. Control monolayers were overlaid with filter sterilized CFS and all the coverslips were incubated for 1 or 2 h at 20° C, rinsed, fixed and examined as above. Each experiment was repeated at least five times.



Figs. 1–3. Mixed haemocyte monolayers after 2 h in vitro. Phase contrast. Scale bars 10 μm

Fig. 1. Haemocytes incubated with HLS-coated *Moraxella* sp. Most cells are lysed. Note the large numbers of bacteria (*B*) associated with the cell remnants

Fig. 2. Haemocytes incubated with sterile CFS. All the cells are intact, many with refractile cytoplasmic granules

Fig. 3. Haemocytes incubated with CFS-incubated *Moraxella* sp. Although intact, most cells have discharged their granules

Fig. 4a–d. Separated haemocytes of *A. astacus*. **a** Usual position of the cell bands in the Percoll gradients. **b** Hyaline cell (phase contrast). **c** Semigranular cell (phase contrast). **d** Granular cell (phase contrast). Scale bars 10 μm

Results

In the mixed haemocyte monolayers, overlaid 2 h previously with HLS-coated *Moraxella* sp., ca. 50–80% of the cells were seen to have lysed (Fig. 1). Similar cell lysis also occurred, within 60 min, in monolayers overlaid with whole, activated HLS in the absence of bacteria, but not in monolayers exposed to CFS or CFS-incubated *Moraxella* sp. alone (Figs. 2, 3). However, with the CFS-incubated bacteria, most of the cells had discharged their granules (Fig. 3). On the experimental (i.e. HLS-coated *Moraxella* sp.) monolayers, ca. 70% of the cells or cell remnants were associated with extracellular bacteria (3–10/haemocyte) (Fig. 1) whereas, in the controls, association between the cells and bacteria was less obvious (0–4 bacteria/haemocyte) (Fig. 3).

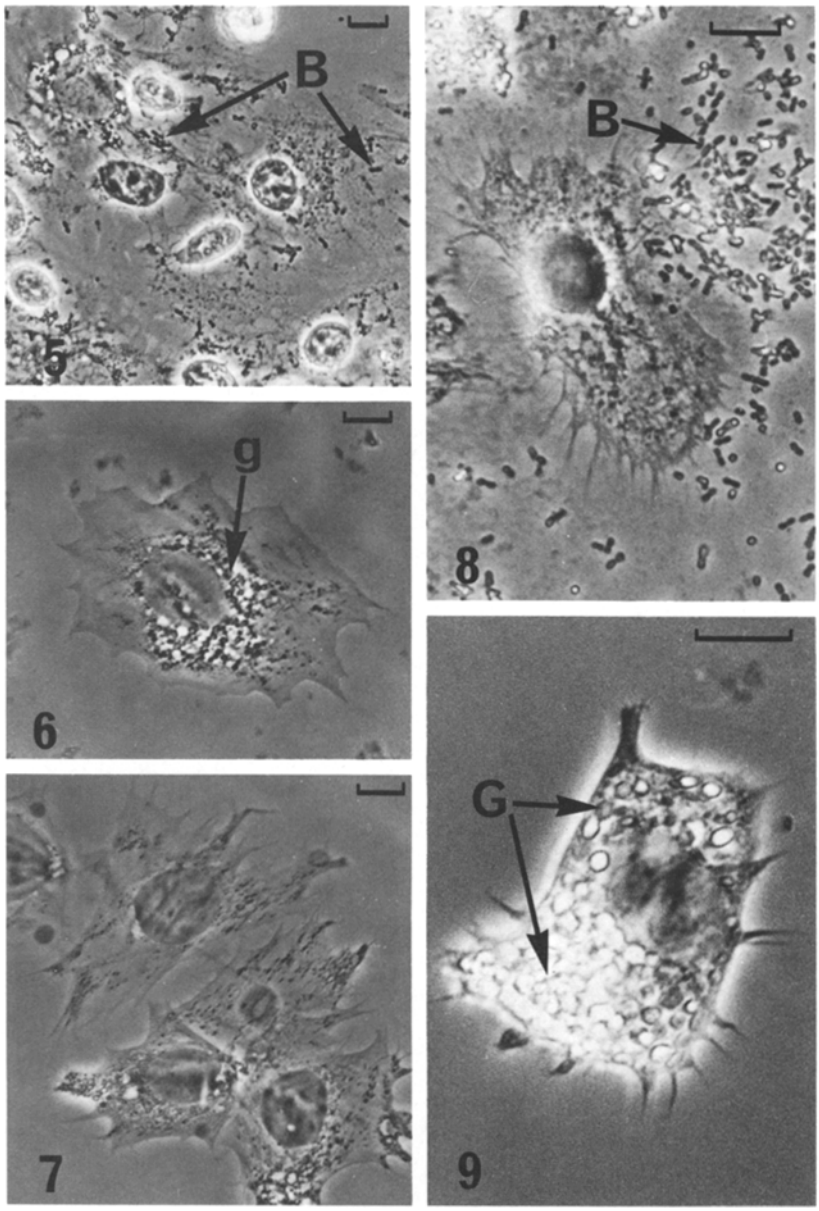
Three haemocyte populations were isolated from *A. astacus* by density gradient centrifugation; the hyaline cells, the semigranular cells and the granular cells (Fig. 4). Morphologically, the cells differed according to the number and size of their cytoplasmic granules (Fig. 4, b–d), and biochemical analyses revealed that phenoloxidase activity was confined to the semigranular and granular cell populations (Table 1). All three cell types were found to attach to the glass surface of the coverslips within 20 min in the presence of calcium (20 mM final concentration) (Table 1) but, as few hyaline cells could be harvested from the Percoll gradients, detailed studies were not made of these cells in vitro. The small number of hyaline cells obtained, however, were seen to attach to and spread slightly on the coverslip surface. Extensive cytoplasmic spreading was exhibited only by the semigranular cells (Table 1).

With the isolated haemocyte populations, incubation of the semigranular cells with the HLS-coated *Moraxella* sp. resulted first in degranulation and then in lysis of ca. 90% of the cells within 1 h (Fig. 5). Large numbers of bacteria were seen to be associated with ca. 75% of the cells and/or cell remnants in the experimental cultures (Fig. 5), although similar association was not detected in the controls (Figs. 6, 7). In the semigranular cell

Table 1. Summary of the characteristics of the different haemocyte populations of *A. astacus* following separation on 70% Percoll in vitro

	Hyaline cells	Semigranular cells	Granular cells
Attachment to glass[a]	+	+	+
Spreading on glass[a]	+	+	–
Phagocytosis of bacteria	NT[b]	+[c]	–
Phenoloxidase activity	–[d]	+	+

[a] Assessed according to the criteria described in Materials and methods
[b] Not tested because of the small numbers of these cells in the Percoll gradients
[c] Ca. 3.0% of the cells after 2 h in vitro, as assessed according to the criteria described in Smith and Ratcliffe (1978)
[d] Phenoloxidase activity, expressed as $\Delta A_{490}\,min^{-1}$, was 0, 0.008 and 0.04 for the hyaline, semigranular and granular cells, respectively



Figs. 5–9. Pure haemocyte monolayers after 1 h in vitro. Phase contrast. Scale bars 10 μm

Fig. 5. Semigranular cells incubated with HLS-coated *Moraxella* sp. Most cells are lysed, but large numbers of bacteria (*B*) are associated with the cell remnants

Fig. 6. Semigranular cell incubated with sterile CFS. The cell is intact and contains cytoplasmic granules (*g*)

Fig. 7. Semigranular cells overlaid with CFS-incubated *Moraxella* sp. The cells are all intact, but have discharged their granules

Fig. 8. Granular cell incubated with HLS-coated *Moraxella* sp. Note the absence of refractile granules in the haemocyte cytoplasm and the large number of bacteria (*B*) on the coverslip

Fig. 9. Granular cell overlaid with CFS-incubated bacteria. The cell contains many large highly refractile granules (*G*)

monolayers overlaid for 1 h with CFS-incubated bacteria, marked degranulation but not lysis of the haemocyte cytoplasm had taken place (Fig. 7). These degranulated semigranular cells resembled the hyaline cells described in an earlier communication (Smith and Söderhäll 1983) so it is possible that the cells we previously identified as hyaline cells may have been degranulated semigranular cells or perhaps, on rare occasions, granular cells. However, we can confirm that the semigranular cells are phagocytic, since, after 2 h in vitro, ca. 3.0% of these cells contained intracellular CFS-incubated *Moraxella* sp. (Table 1), as assessed according to the criteria outlined in Smith and Ratcliffe (1978). The granular cells, by contrast, were never seen to enclose bacteria (Table 1), and after 1 h incubation with the HLS-coated *Moraxella* sp. underwent degranulation but not lysis (Fig. 8). Degranulation was not apparent in the granular cells inoculated with the CFS-incubated *Moraxella* sp. after 1 h (Fig. 9), nor the monolayers treated with saline alone.

Discussion

Comparison of the haemocytic reactions of *A. astacus* in vitro to HLS-coated and CFS-incubated bacteria revealed an extreme degree of degranulation and cell lysis on the experimental coverslips within 1 h. With the separated haemocyte populations, the semigranular cells were found to degranulate upon exposure to the bacteria alone, but to lyse, after degranulation, during incubation with the HLS-coated *Moraxella* sp. In contrast, the granular cells degranulated, without lysis, only in the presence of the HLS-coated *Moraxella* sp. Granule discharge and haemocyte lysis are known to occur during the early stages of capsule and nodule formation in vivo in insects (Schmit and Ratcliffe 1977, 1978; Ratcliffe and Gagen 1977), and crabs (Smith and Ratcliffe 1980), as well as to foreign materials in vitro (Ratcliffe 1975; Smith and Ratcliffe 1978). Furthermore, Armstrong and Rickles (1982) have recently shown that degranulation is induced in *Limulus* amoebocytes in vitro by lipopolysaccharides, and we have reported degranulation of *A. astacus* haemocytes following in vitro treatment with β1,3-glucans (Smith and Söderhäll 1983). Degranulation must, therefore, represent an important cellular reaction to foreignness, probably facilitating release of previously cell-bound recognition factors (prophenoloxidase activating components) into the haemolymph. These released cell proteins probably then trigger adjacent haemocytes to degranulate, particularly as, in the present paper, whole HLS, in the absence of bacteria, was observed to induce degranulation of the cells on the coverslips, and Söderhäll and Häll (unpublished) have found that the prophenoloxidase cascade in haemocyte lysate preparations is activated by HLS proteins pre-converted from self to non-self. That the granular cells were induced to discharge their granules only by the HLS-coated bacteria, and not by the saline-incubated control *Moraxella* sp., indicates that the attaching proteins in haemocyte lysate provide stronger non-self signals than the bacterial cell walls. However, we are unable to explain, at present, the reason for the lysis of the

semigranular cells on the experimental coverslips. Possibly, this may have been due to the stress imposed on the cells, under the in vitro conditions, by these strong non-self signals or, alternately, have been caused by a direct cytotoxic action of phenoloxidase and the other sticky proteins. Since preliminary experiments (Wingren and Söderhäll, unpublished) have failed to reveal a cytotoxic effect of crayfish HLS against ^{51}Cr-labelled tumour cells, the latter suggestion seems unlikely.

The in vitro monolayer technique is widely used by invertebrate immunologists to study phagocytosis and detect opsonins without the inherent complications of the in vivo state. By employing β1,3-glucans we have recently used this technique to demonstrate, indirectly, that the prophenoloxidase activating system contains opsonic factors. Unfortunately, in our present study, because of the more rapid degranulation induced in the haemocytes by the HLS proteins, attempts were not made to confirm the role of the attaching proteins as opsonins. However, enhanced recognition by crayfish haemocytes has been achieved in vivo by coating fungal spores with the attaching proteins in HLS (Söderhäll et al. 1983) and this response resulted in the rapid formation of large cell clumps rather than the ingestion of the fungal spores by the haemocytes. In the present in vitro investigation, we were able to see increased adherence of bacteria to the cells or the cell remnants on the experimental coverslips, so that the attaching proteins of the prophenoloxidase system do appear to assist in the non-specific entrapment of foreign particles by the cells. Nonetheless, in view of the findings recorded in the present paper, together with those of Armstrong and Rickles (1982) on *Limulus*, and Smith and Söderhäll (1983) on crabs and crayfish, it is clear that caution should be exercised in identifying individual cell types on the basis of their morphological features alone. Moreover, since crustacean haemocytes are so sensitive to strong non-self stimuli, the use of the monolayer technique may be inappropriate for evaluation of the role(s) of the various haemolymph proteins as opsonins.

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