Selected decrease of haemocytes of the freshwater snail *Planorbarius corneus* (L.) (Gastropoda, Pulmonata) after bacteria injection

E. Ottaviani

Department of Animal Biology, University of Modena, via Berengario 14, I-41100 Modena (Italy)
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Summary. The decrease of haemolymph phagocytic cells (SH) in *Planorbarius corneus* after bacterial injection seems to be mediated by humoral factor(s) released into the haemolymph. SH show different adhesiveness in vitro in the presence of bacterial metabolic products.
Key words. Planorbarius corneus; *Staphylococcus aureus*; haemocyte decrease factor(s).

Bacterial injection in molluscs causes a rapid fall in the number of circulating haemocytes[1-3]. This effect also occurs in insects[4-6]. A decrease of the main circulating phagocytic cells has been observed in *Galleria mellonella*. This drop is mediated via the release of a humoral factor by plasmatocytes in response to *Bacillus cereus* injection[7,8].

This paper reports a similar finding following bacterial injection in the mollusc *Planorbarius corneus*.

Materials and methods. Adult specimens of *Planorbarius corneus* (L.) were injected with 10 µl of *Staphylococcus aureus* (15×10^6 living bacteria/ml) grown in nutrient broth, as previously described[9]. 100 µl of haemolymph were taken from each animal (n = 6) after 15, 30, 60 and 120 min. The haemolymph was subsequently cytocentrifuged on slides and stained with May-Grünwald and Giemsa (MGG). The same procedure was carried out in animals (n = 6) injected with 10 µl of snail saline solution[10].

In a second experiment, another group of animals (n = 6) was injected with 15 µl of cell-free haemolymph; the procedure described above was then followed. Cell-free haemolymph was obtained from haemolymph collected from three snails 30 min after injection with 10 µl of *S. aureus* (14×10^6 bacteria/ml). The haemolymph was centrifuged at 1200 × g for 10 min. Two aliquots of the supernatant were utilized: 15 µl was injected into the snails, as mentioned above; the remaining amount was used on petri dishes to test for the presence of *S. aureus*. The haemolymph cell-type percentages present in the specimens used in two experiments were calculated on at least 200 cells for each animal. Figure 1 reports the results expressed as mean percentage ± standard deviation.

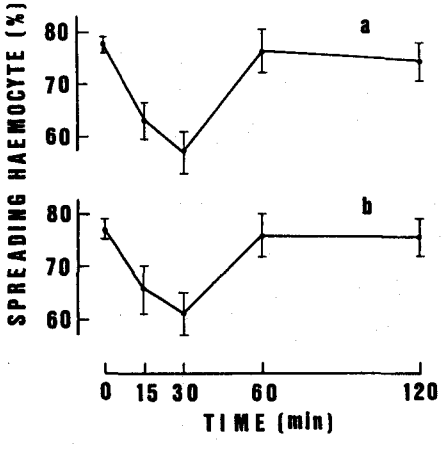

Figure 1. Percentage of spreading haemocytes out of total blood cell count: *a* after bacterial injection, *b* after cell-free haemolymph injection.

In vitro cellular adhesion under different conditions was also studied. Test conditions involved: 1) haemolymph (from a single specimen) and nutrient broth alone, 2) haemolymph and nutrient broth containing *S. aureus* (10×10^6 bacteria/ml), or 3) haemolymph and nutrient broth containing normal bacterial metabolic products. Nutrient broth with normal bacterial metabolic products alone was obtained by centrifuging (1200 × g, 10 min) the nutrient broth used to grow *S. aureus* for 24 h at 37 °C. An aliquot of supernatant was cultured on petri dishes to test for the presence of bacteria; the rest was used in the experiment. The following method was applied for all three tests: 100-µl samples of nutrient broth obtained under the conditions described above, were added to 100 µl of haemolymph in a plastic tube. The tubes were placed in a revolving mixer for 10 min, and the resulting mixture was left in a petri humidified chamber for 30 min at room temperature. The supernatant was then discarded, and the cells were dried and stained with the MGG technique.

Results and discussion. Specimens of *P. corneus* injected with *S. aureus* showed a rapid and selective removal of spreading haemocytes (SH), one of the two cell types present in the circulating haemolymph[10,11]. The SH are responsible for phagocytosis[9-11]. As illustrated in figure 1 a, SH percentage decreases from 78% at time zero to 57% after 30 min, returning to initial values after 60 min. A similar behaviour was observed when cell-free haemolymph was injected into *P. corneus* (fig. 1 b). Indeed, the drop in SH spans 60 min, with the greatest decrease (61%) after 30 min. The decrease in SH percentage is less pronounced in specimens injected with cell-free haemolymph. It is important to note that cell-free haemolymph cultures did not reveal *S. aureus* growth, and thus the depletion in SH is likely to be due to the presence of humoral factor(s). In insects, this response

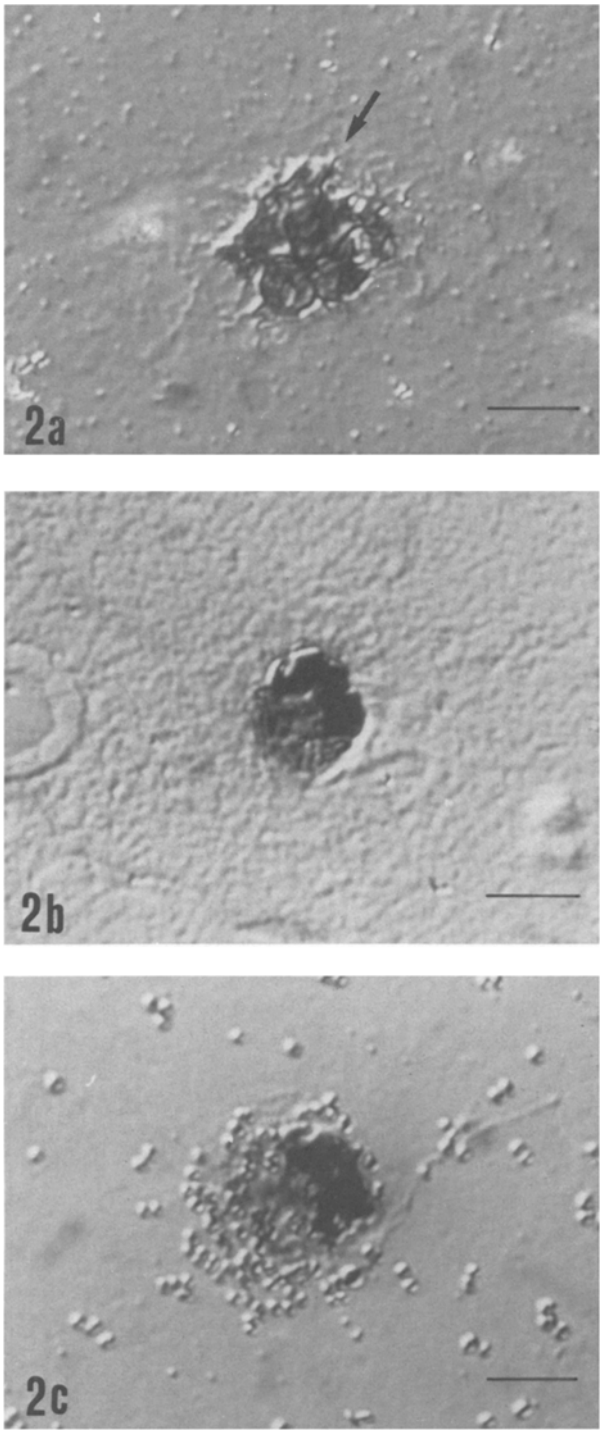

Figure 2. Spreading haemocyte morphology: *a* following contact with bacterial metabolic products (arrow indicates a pseudopod); *b* following contact with nutrient broth alone; *c* Phagocytosis of *Staphylococcus aureus* by spreading haemocyte (Nomarski optics), bar = 100 µm.

has been interpreted as the first step in the inflammatory reaction. In *G. mellonella,* the presence of this depletion factor increases the adhesiveness of plasmatocytes, and this can cause the removal of blood cells from the circulating haemolymph. Adhesion of plasmatocytes to the solid substratum of the haemocoel lining could allow for chemotactic migration towards bacteria [8].

A mechanism similar to that observed in *G. mellonella* could take place in the cellular defence reaction against bacteria in *P. corneus*. This is supported by both the decrease in SH and the results of in vitro cellular adhesion tests. Indeed, tests performed under the different conditions showed that only one of the two cell types, i.e. SH adhere to glass. Blood cells coming into contact with bacterial metabolic products underwent marked changes in cell morphology, such as the extension of numerous pseudopods (fig. 2a). These modifications are less evident in blood cells mixed and incubated in nutrient broth alone (fig. 2b). Lastly, the normal functional status of SH was confirmed by the fact that phagocytosis was observed in nutrient broth containing bacteria (fig. 2c). The different cellular morphology revealed by consequent changes in adhesiveness of the SH after incubation may be due either to chemotactic activity of bacterial metabolic products and/or to the depletion factor(s) released from SH, as suggested by the in vivo tests.

Bacterial clearance studies in *Helix pomatia* [1] and *Lymnaea stagnalis* [3] show a rapid and marked drop in circulating amoebocytes during the initial phase of the clearance process. This decrease has been attributed to blood cell clumping and increased adhesion to the walls of blood vessels. Furthermore, migration of amoebocytes into the connective tissue has been observed in *L. stagnalis*.

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