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Abstract. The lectin wheat-germ agglutinin (WGA) selectively binds N-acetyl-D-glucosamine. Fluorescence and electron microscopy were used to show that WGA stains the cytoplasmic granules in the granulocytes, but not the hyaline cells, of two decapods, the ridgeback prawn Sicyonia ingentis and the American lobster Homarus americanus. Using fluorescence microscopy, two intermediate stages in granulocyte maturation were observed. Cells smaller than typical small-granule hemocytes were observed with 5 or fewer granules, which in previous studies using brightfield and phase optics were probably counted as hyaline cells. Also, some granulocytes were observed containing both small and large granules, supporting the suggestion that small and large granule hemocytes represent stages in the maturation of one cell line. Granules in the single type of hemocyte in the branchiopod Artemia franciscana did not stain with WGA. The possible roles of N-acetyl-D-glucosamine in wound healing, pathogen encapsulation, and maintenance of normal crustacean connective tissues are discussed.

Additional key words: hemocytes, crustaceans, wheat-germ agglutinin (WGA), N-acetyl-D-glucosamine, chitin

The exoskeleton of arthropods forms a physical barrier against infection. Pathogens that can penetrate the thin, waxy epicuticle face the bulk of the exoskeleton composed of thick layers of protein and chitin (Söderhäll & Unestam 1975; Johnson 1980; Dall et al.1990; Gibbs 1998; O’Brien et al. 1991). Chitin is composed of polymers of N-acetyl-D-glucosamine and procedures for the identification of this molecule have been reviewed by Peters and Latka (1986). Most techniques require tissue digestion whereas the selective labeling of chitin by a lectin from Triticum vulgaris (commonly known as wheat germ agglutinin, WGA) has been used to localize chitin in the peritrophic membrane of insects (Lehane et al. 1996; Harper & Hopkins 1997), and the radula sac of the gastropod Biomphalaria glabrata (Peters & Latka 1986). The specificity of WGA labeling is enhanced by using succinylated WGA which has a higher binding affinity (Wagner 1994; Monsigny et al. 1979), and employing competitive inhibitors such as N-acetyl-D-glucosamine and triacetyl chitotriose (Peters & Latka 1986).

Hemocytes (i.e., circulating blood cells) are a second line of defense against infections in crustacea. They initiate hemolymph coagulation, which prevents dissemination of pathogens around the body, as well as phagocytosing and encapsulating foreign materials (Martin & Hose 1992). Classification schemes for crustacean hemocytes have relied heavily upon the presence or absence of cytoplasmic granules. Whereas hyaline cells and granulocytes in most decapods can be identified by the absence or presence of granules, respectively, the hyaline cells in some decapods contain obvious granules (Hose et al. 1990; Martin and Hose 1992). Therefore, hemocyte classification should be based on cell morphology supported by cytochemical and functional studies. Work in this lab (Hose et al. 1990; Martin & Hose 1992) has suggested the following criteria for identifying decapod hemocytes. Hyaline cells are small with a large nuclear/cytoplasmic (N/C) ratio, lyse on contact with seawater to initiate coagulation of the hemolymph, and contain few lysosomes and no phenoloxidase. Granulocytes are larger cells with smaller N/C ratios, contain lysosomal enzymes and prophenoloxidase, and phagocytose and/or encapsulate foreign materials. Granulocytes may be subdivided morphologically into those containing...
small or large cytoplasmic granules, but there is no evidence of cytochemical or functional differences in these cells. Additional criteria should help the ongoing efforts to identify and properly classify crustacean hemocytes.

During an investigation into the structure of the midgut trunk in the shrimp, *Sicyonia ingentis*, it was noticed that some hemocytes stained with WGA. In this paper we 1) demonstrate the presence of N-acetyl-D-glucosamine within the granules of hemocytes in two decapods but not in the single granulated type of hemocyte in the branchiopod *Artemia franciscana*, 2) apply this information to hemocyte classification, and 3) discuss possible functions of this molecule.

**Methods**

Three crustaceans were examined in this study; ridgeback prawn *Sicyonia ingentis* BURKENROAD 1938, American Lobster *Homarus americanus* MILNE EDWARDS 1837, and brine shrimp *Artemia franciscana* KELLOGG 1906. Prawn were collected by otter trawls at a depth of 130 m off the coast of Palos Verdes peninsula, Southern California. Lobsters and brine shrimp were purchased at local fish markets and tropical fish stores, respectively.

Hemolymph (0.5 mls) was collected from the ventral sinus of prawn and lobsters and added to 1.5 ml of 5% paraformaldehyde in Amersham phosphate buffered saline (PBS) pH 7.4. The mixture was pipetted onto glass slides previously coated with poly-L-lysine and the cells were allowed to settle and bind to the slides for 1 hr at room temperature. Hemocytes from brine shrimp were collected by severing the bodies of 5–10 animals in a drop of fixative on a glass slide previously coated with poly-L-lysine. Slides with adherent hemocytes were washed twice in PBS, stained for 1 hr in the dark with 50 μl of FITC labeled WGA (Sigma L4895), washed in PBS and viewed by bright field and fluorescence microscopy. WGA at concentrations as low as 10 μg/ml, a concentration of 25 μg/ml was routinely used because the fluorescence was more intense and allowed for photography and multiple viewing before quenching. Staining with succinylated WGA demonstrated the same staining pattern. TEM also showed the same pattern of hemocyte labeling as seen at the LM level, and that WGA-binding was localized to the cytoplasmic granules of decapod granulocytes (Fig. 3).

**1. Staining Patterns**

No fluorescence was observed in unfixed hemocytes from all 3 species, nor in hemocytes fixed in either paraformaldehyde or glutaraldehyde. Following incubation with WGA, small- and large-granule hemocytes in both *H. americanus* and *S. ingentis* showed bright fluorescent cytoplasmic granules (Figs. 1c & e), whereas neither the hyaline cells of these decapods (Fig. 1a) nor the granules in the single type of hemocyte circulating in *A. franciscana* (Figs. 2a & b) showed any fluorescence. Although granules in the granulocytes of *H. americanus* and *S. ingentis* could be seen fluorescing after treatments with WGA at concentrations as low as 10 μg/ml, a concentration of 25 μg/ml was used because the fluorescence was more intense and allowed for photography and multiple viewing before quenching. Staining with succinylated WGA demonstrated the same staining pattern. TEM also showed the same pattern of hemocyte labeling as seen at the LM level, and that WGA-binding was localized to the cytoplasmic granules of decapod granulocytes (Fig. 3).

**2. Hemocyte Classification**

Approximately 2000 hemocytes treated with WGA-FITC were classified from each of the three species of crustacea examined. WGA-staining of hemocytes in *Artemia salina* revealed a single type of cell containing cytoplasmic granules that did not fluoresce. Hemocytes from *S. ingentis* and *H. americanus* were placed into one of the following 5 categories based on their general morphology and staining pattern with WGA. Three of these categories were the traditional standards; hyaline cells, small-granule, and large-granule hemocytes, and two intermediate categories were added. Hyaline hemocytes (Fig. 1a) were identified as small cells with only a thin rim of cytoplasm around the nucleus and they showed no fluorescence. Type A hemocytes (Fig. 1b) resembled hyaline cells but had more cytoplasm and contained 1–5 tiny fluorescent granules. Small granule hemocytes (Fig. 1c) had abundant cytoplasm containing numerous, brightly fluo-
Figs. 1–3. Fig. 1A–E. Fluorescence micrographs of hemocytes from *S. ingentis* stained with FITC-WGA. Hyaline cells (Fig. 1A) lack fluorescing granules. Type A cell (Fig. 1B) are slightly larger than hyaline cells and contain a few fluorescent granules (arrows). Small- (Fig. 1C) and large-granule (Fig. 1E) hemocytes contain numerous fluorescing cytoplasmic granules. Type B hemocytes (Fig. 1D) have large (L) and small (S) granule within the same cell. (Both granules have maximum diameter in the same focal plane.) Scale bar = 5 μm. Figs. 2A & B. Granulocytes from the brine shrimp *A. franciscana* stained with FITC-WGA and examined by phase contrast (Fig. 2A) and fluorescence microscopy (Fig. 2B). Note the large granules (arrow) filling the cytoplasm in the phase image which do not react with WGA-FITC. Scale bar = 5 μm. Fig. 3. TEM of large-granule hemocyte from *S. ingentis* showing cytoplasmic granules (G) labeled with WGA bound gold particles (black dots). N, nucleus. Scale bar = 1 μm.
Table 1. Comparison of differential hemocyte counts based on Light and WGA-FITC fluorescence microscopy.

<table>
<thead>
<tr>
<th></th>
<th>% Hyaline hemocytes</th>
<th>% Type A hemocytes</th>
<th>% Small granule hemocytes</th>
<th>% Type B hemocytes</th>
<th>% Large granule hemocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sicyonia ingentis</em></td>
<td>LM 54</td>
<td>0</td>
<td>36</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>WGA-FITC 45</td>
<td>18</td>
<td>25</td>
<td>4</td>
<td>8</td>
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<tr>
<td><em>Homarus americanus</em></td>
<td>LM 16</td>
<td>0</td>
<td>60</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>WGA-FITC 15</td>
<td>17</td>
<td>22</td>
<td>20</td>
<td>26</td>
</tr>
<tr>
<td><em>Artemia franciscana</em></td>
<td>LM 0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>WGA-FITC 0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

rescuing granules. Type B hemocytes (Fig. 1d) contained a mixture of small and large granules and in large granule hemocytes (Fig. 1e) the abundance of exclusively large sized granules made the entire cell fluoresce brightly. The percentages of these types of hemocytes are shown in Table 1 along with percentages of hyaline, small-granule, and large-granule hemocytes previously reported in the literature from our lab for each of these species (Hose & Martin 1989; Omori et al. 1989; Hose et al. 1990).

**Discussion**

In this paper, we have demonstrated that granules in the granulocytes of two decapods, the shrimp *S. ingentis* and the lobster *H. americanus*, react with WGA, whereas there is no labeling in either the hyaline cells in these decapod crustaceans or the granulocytes in the brine shrimp *A. franciscana*. The presence of N-acetyl-D-glucosamine adds to the list of materials known to be contained within the cytoplasmic granules of decapod hemocytes, including lysosomal enzymes and phenoloxidase (Hose et al 1987; Hose and Martin 1989; Söderhall & Smith 1983), as well as penaeidins, antimicrobial peptides with chitin-binding activity (Destoumieux et al. 2000). N-acetyl-D-glucosamine may be present as either chitin (Peters & Latka 1986) or glycosylated proteins (Alberts et al. 1989) and may contribute to the normal connective tissue or the extracellular matrix formed during wound healing or pathogen encapsulation.

The differential labeling of crustacean hemocytes by WGA may be useful in identifying hemocytes. Decapods are generally regarded as having three types of circulating hemocytes (Martin & Hose 1992). In the two decapods we examined, the granules in all granulocytes showed reaction sites for WGA. Hyaline cells in *S. ingentis* did not bind WGA even though these cells contain a few, small granules. Hyaline cells in lobsters also contain a small number of granules, which are obvious using bright field and phase contrast microscopy. These granules also did not react with WGA whereas there was intense binding of WGA to the small and large granules in granulocytes of both decapods. The differential binding of WGA to hyaline cells and granulocytes is consistent with our (Hose et al. 1990; Martin & Hose 1992) suggestion that these cell types represent distinct cell lineages but does not rule out the alternative possibility of a single line of hemocyte maturation.

A comparison of differential hemocyte counts based on WGA-FITC staining and previously published studies using bright field and electron microscopy, show general similarity. In performing differential counts using WGA-FITC we kept the traditional categories of circulating hemocytes (hyaline cells, small-granule and large-granule hemocytes) and added two more (types A and B). Type A cells were smaller than traditional small-granule hemocytes and showed less than 5 small fluorescent spots indicating cytoplasmic granules. We suggest that these cells represent early stages in the development of small granulocytes. Considering the small number and size of these granules in shrimp, it is not surprising that these cells were probably regarded as hyaline cells and the granules as mitochondria resulting in an inflation of the actual differential count of hyaline cells. The granules in the lobster hyaline cells are larger and the suite of morphological, cytochemical and functional criteria used to recognize these cells (Hose et al. 1990) more accurately identified them. The second new category, type B granulocytes, was established for WGA-FITC stained cells which contained a mixture of small and large fluorescing granules. We interpret these images as representing hemocytes maturing within the granulocyte lineage from small to large granule cells. In the shrimp, only 4% of hemocytes showed this combination whereas it was more common (20%) in the lobster. This may reflect a different rate of maturation in the two species or different physiological conditions of the animals used in this study.

We do not propose that the two new terms used in this paper be added to the current nomenclature of
decapod hemocytes. We used the terms to highlight what we interpret as two new stages in the maturation of granulocytes that are more easily observed using the WGA-FITC technique instead of the standard bright field or phase image. It is also important to note that monoclonal antibodies are being developed to recognize molecules on and in hemocytes which may improve our classification schemes (Bachère et al. 1995; Rodriguez et al. 1995). Sung and Sun (2002) used antibodies to four surface molecules to identify six categories of hemocytes in the penaeid shrimp Peneaus monodon. Further work is necessary to identify markers that will facilitate our understanding of hemocyte function.

Finally, little is known about the hemocytes in non-decapod crustaceans, which may have 0–3 or more types of circulating cells (Barracco & Amirante 1992; Benjamin & James 1987; Boxshall 1992; Schmitz 1992; Wagele 1992; Walker 1992). Previous studies on the branchiopod A. franciscana suggested that they have a single type of hemocyte filled with large cytoplasmic granules (Lochhead & Lochhead 1941; Martin et al. 1999). These observations are supported in the present study by the fact that none of the circulating cells stained with WGA. Since N-acetyl-D-glucosamine is absent in the single branchiopod we tested and present in the two decapods we studied, it would be useful to determine its distribution in hemocytes and connective tissues of other crustaceans. WGA has also been used in studies on the classification and function of insect hemocytes (Hillyer & Christensen 2002). Further comparative studies may help provide information on properties of crustacean hemocytes and their involvement with connective tissues in normal and disease conditions.

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References


