Patterns of Hemocyte Production and Release Throughout the Molt Cycle in the Penaeid Shrimp *Sicyonia ingentis*

JO ELLEN HOSE*, GARY G. MARTIN, SAM TIU, AND NANCY MCKRELL

Biology Department, Occidental College, 1600 Campus Road, Los Angeles, California 90041

Abstract. The production and release of hemocytes was evaluated throughout the molt cycle in the shrimp *Sicyonia ingentis*. Hematopoiesis occurs in paired epigastric hematopoietic nodules (HPN) which consist of an extensive network of vessels. Hemocytes are produced within the walls of these tubules and released into the vessel lumens. During molt stage C (intermolt), few cells were present in the tubule wall; most of these were hematopoietic stem cells. Elevated mitotic rates during stages C to D_{1-2} (2–4%) led to the production and rapid release of individual hemocytes, primarily granulocytes. Although the mitotic rate progressively declined from stage D_{1-4} until after ecdisis (stage A_{1}), the maturing hemocytes accumulated within the tubule walls. Around ecdisis, production of hyaline hemocytes exceeded that of granulocytes. Large groups of these hemocytes were channeled into the vessel lumens immediately after molting. Mitotic rates increased again during stages A_{2} and B with the number of hemocytes in the tubules reaching seven times that of stage C.

Morphological stages in the transition of hematopoietic stem cells into hyaline hemocytes and granulocytes are described, and a model of decapod hemocyte maturation is presented.

Introduction

Decapod hemocytes are produced within specialized hematopoietic tissue, the location and architecture of which vary greatly, even within close taxonomic groups. In lobsters, crabs, and crayfish, a sheet-like hematopoietic tissue covers the cardiac stomach or the heart, and maturing hemocytes are organized into small lobules of variable thickness (Cuénot, 1893, 1905; Kollman, 1908; Demal, 1953; Ghiretti-Magaldi et al., 1977). Stem cells line the apical border of each lobule and maturing hemocytes migrate toward the hemal space (Johnson, 1980). Young hemocytes are liberated into adjacent hemal spaces and then into the general circulation. Paired epigastric hematopoietic nodules (HPN) are present in penaeid shrimps, (Oka, 1969; Martin et al., 1987) and some species may have ancillary sites of hematopoietic tissue surrounding the antennal artery, and at the base of the maxillipeds (Bell and Lightner, 1988). For this study, a penaeid shrimp (*Sicyonia ingentis*) was chosen because it has a simple hematopoietic system; hematopoiesis occurs only within the HPN as ancillary sites have not been demonstrated. The HPN is formed by extensive branching of the ophthalmic artery, and hemocytes are produced within the wall of these vessels, termed hematopoietic tubules. The lumen of each tubule is lined by endothelial cells, through which most hemocytes enter the peripheral circulation. Hemocytes and stem cells are located within the fibrillar extracellular matrix, which comprises the wall of the tubule. The outside of the tubule is surrounded by capsular cells, which apparently secrete a dense meshwork of collagen fibrils. Hemocytes exit through the outer wall into the open circulatory system less frequently (Martin et al., 1987).

The regulation of hematopoiesis in decapod crustaceans is poorly understood, but is probably influenced by physiological processes such as molting, reproduction, and disease status, as well as by environmental conditions like temperature and water quality (Baucuch and Plaquet, 1973; Johnson, 1980; Bauchuch, 1981). Detailed information on the effect of the molt cycle on hemocyte production and release is not available. Two studies (Marrec,
1944; Charmantier, 1972) showed that mitotic activity in hematopoietic tissue of crabs was high during the intermolt period (stages late B and early C) and low immediately after molting (stage A). The major release of hemocytes occurred during the ecdysial interval (stages D through A) (Marrec, 1944; Johnson, 1980).

Studies published on hematopoeisis have not attempted to classify the maturing hemocytes and stem cells. Such distinctions are essential because the different categories of hemocytes perform different functions (Hose et al., 1990). Most researchers divide decapod hemocytes into two major groups, hyaline hemocytes and granulocytes (Bauchau, 1981; Hose et al., 1990). Hyaline hemocytes initiate coagulation of the hemolymph (Omori et al., 1989) and are thought to be involved in hardening (tanning) of the exoskeleton after molting (Vacca and Fingerman, 1983). In contrast, granulocytes are responsible for defense against foreign materials (Ratner and Vinson, 1983; Söderhäll et al., 1988; Hose et al., 1990) and may liberate proteins which contribute to the formation of the exoskeleton (Vacca and Fingerman, 1983). Identification of early maturation stages can enhance our understanding of hemocyte genesis particularly regarding whether granulocytes and hyaline hemocytes constitute a single line or two separate lines of maturation.

The dynamics of hemocyte production and release were followed throughout the molt cycle in the shrimp *Sicyonia ingentis*. Hematopoietic nodules were removed at various stages of the molt cycle, and, mitotic activity, hemocyte maturation, and release were quantified. Hemopoietic cells were categorized using morphological criteria corresponding to cytochemical and physiological properties of shrimp hemocytes (Martin and Hose, in press). The production and release of granulocytes could, therefore, be differentiated from that of hyaline hemocytes. In addition, the ultrastructure of stem cells and maturing hemocytes were described, contributing morphologic evidence for a model of decapod hemocyte maturation. The information presented here is expected to form a basis for future studies utilizing physiological and biochemical techniques.

**Materials and Methods**

*Animal collection*

Shrimp were collected in 160 m of water off Huntington Beach, California and maintained in aquaria with flow-through seawater at 18°C. They were fed *ad libitum* on a commercial shrimp diet.

*Molt staging*

Molt stage was determined by observing the retraction of epithelium within setae of the antennal scales (Anderson, 1985). Shrimp were categorized into one of the following molt stages: A₁, A₂, B, C, D₀, D₁₋₂, and D₃₋₄.

**HPN preparation**

Hematopoietic nodules (Martin et al., 1987) were dissected and immediately fixed in 2.5% glutaraldehyde in a 0.1 M solution of sodium cacodylate at pH 7.8 containing 12% glucose for 2–3 hrs at 20°C. Tissues were washed in a 0.1 M solution of sodium cacodylate at pH 7.8 containing 24% sucrose for > 10 min and then post-fixed for 1 h in 1% OsO₄ in a 0.1 M solution of sodium cacodylate at pH 7.8. The nodules were stained en bloc for 1 h in 3% uranyl acetate, then dehydrated, infiltrated and embedded in Spurrs’ (1969) resin and sectioned. Thick sections (0.5 μm) were routinely stained in methylene blue for examination under an Olympus light microscope (LM). Thin sections (90 nm) were stained in Reynolds lead citrate for 5 min and viewed using a Zeiss EM 109 transmission electron microscope (TEM). Five animals were processed and analyzed for each molt stage.

**LM analyses**

*Tubule counts.* Cells for each shrimp were counted and identified in cross sections of four tubules. Lumen diameter and tubule wall width were measured using an ocular micrometer. Cell counts could be directly compared because mean widths of the lumen and wall did not differ significantly throughout the molt cycle or between animals. In one half of each tubule and the corresponding lumen, all of the hemocytes and stem cells were counted and categorized into one of the following: hyaline stem cells, hyaline hemocyte, granulocyte stem cell, small granule stem cell, small granule hemocyte, or large granule hemocyte. The basic characteristic used to distinguish between cells of the hyaline and granulocyte lines was the relatively darker cytoplasmic staining of the former (greater electron density for TEM evaluations); this characteristic was visible in every cell regardless of the angle of sectioning. Stem cells had boundaries that were difficult to resolve using LM; on TEM examination, this appeared to result from their tight apposition to other cells. In contrast, hemocytes were separated from adjacent cells, usually by fibrillar extracellular matrix (Fig. 1c, d, f) (Martin et al., 1987). Hemocytes had rounded contours or were migrating through the tubule wall. Morphological descriptions were based on examination of at least 50 oblique sections of cells with a nucleus. Longest axes were measured in at least 20 of these cells.

Hyaline hemocytes were distinguished from hyaline stem cells on the basis of their smaller size, higher N:C ratio, and their densely basophilic nucleus. Hyaline stem cells were separated from granulocyte stem cells because the former were smaller, had cytoplasm, which stained
Figure 1. Transmission electron micrographs and light micrographs (inserts) of cells from the hematopoietic tissue of shrimp. (A) Early granulocyte stem cell, (B) more mature granulocyte stem cell, (C) small granule hemocyte, (D) large granule hemocyte, (E) hyaline stem cell, and (F) hyaline hemocyte. All figures at 8000×; scale bar = 2.5 μm. Light micrographs—methylene blue, 1800×.
darkly with methylene blue, and contained at most two cytoplasmic granules. At the LM level, granulocyte stem cells possessed pale blue cytoplasm with zero to two obvious granules and a distinctive nucleus containing finely granular chromatin and one or two large nucleoli. The small granule stem cells had a lower N:C ratio than the granulocyte stem cells, their nuclei were more basophilic with smaller nucleoli, and they contained between three and ten granules. Small granule hemocytes were separated from the granulated stem cells on the basis of their oval shape, numerous small (0.4 μm diameter) dark cytoplasmic granules, and lower N:C ratio. Large granule hemocytes had, in addition to small cytoplasmic granules, large cylindrical (0.8 μm in longest diameter) granules and the lowest N:C ratio of any cell type observed. Only nucleated cells were counted. Hemocytes exiting the tubule via the endothelium or outer margin were recorded. Capsular cells on the outside of the tubule were not enumerated.

Cell counts from four tubules were averaged to obtain profiles for each animal. Numbers and proportions of hyaline stem cells, hyaline hemocytes, granulocyte and small granule stem cells, and small and large granule hemocytes were calculated, and values from the five animals were averaged. Longest axes of stem cells were measured using an ocular micrometer. Hemocytes within the tubule wall were not measured because many were irregularly shaped as they migrated into the lumen. Cells within the lumen were similarly evaluated, and diameters of hyaline and small granule hemocytes were measured. Means of these parameters were compared throughout the molt cycle. Where appropriate, data were evaluated for differences using t-tests or analysis of variance with a significance level set at P = 0.05.

Mitotic index. Dividing cells were recorded and identified during LM analyses of the tubules. Hematopoietic cells were considered mitotic from the disappearance of the nuclear membrane during prophase to its reappearance during telophase. The absence of the nuclear membrane was observed at both the LM and TEM levels. Nuclear characteristics were not used to identify dividing cells because of nuclear changes occurring during mitosis. Instead, cells of the hyaline and granulocyte lines were distinguished by the relatively darker cytoplasm of the hyaline cells using LM (greater cytoplasmic electron density using TEM). To further strengthen the assignment of a cell as hyaline or granulocyte, the darkness of its cytoplasm was usually compared to that of non-dividing cells adjacent to it which could be identified as hyaline or granulocyte based on characteristics of the nucleus and cytoplasmic granules. Using criteria described in the previous section, a dividing cell was considered to be a stem cell if its plasma membrane was contiguous with other cells, and a hemocyte if it was detached. To determine the mitotic index, the number of mitotic hemocytes/precursors was divided by the total number of hematopoietic cells examined within the tubule. Endothelial cells and hemocytes within the lumen were not included in these calculations although mitotic activity was recorded. Percentages of mitotic hemocytes in each category were then calculated.

TEM analyses

Transmission electron microscopy (TEM) was used to (1) verify the identity of cells tentatively identified using LM, (2) describe the ultrastructure of maturing hemocytes, and (3) determine nucleocytoplasmic (N:C) ratios of stem cells and maturing hemocytes. Nucleocytoplasmic ratios on longitudinal sections of cells were determined by dividing the area of the nucleus by the area of the cell. Measurements were made using electron micrographs placed on a digitizing tablet and were analyzed by Sigma-Scan® computer software (Jandel Scientific).

Results

Description of stem cells and maturing hemocytes

Cells that were sectioned through the nucleus could be classified into one of six categories: granulocyte stem cell, small granule stem cell, small granule hemocyte, large granule hemocyte, hyaline stem cell, and hyaline hemocyte (Fig. 1). Granulocyte stem cells (Fig. 1a) were large, averaging between 7.1 and 9.9 μm in longest diameter (Table I). The cell outline was irregular and the plasma membrane often difficult to discern. These cells had a very high N:C ratio (65.8% ± 8.8%, n = 10; X ± SD) and the nucleus was frequently oval with only a thin rim of margined chromatin visible. The single large nucleolus (1.5 μm diameter) was the most distinctive feature visible at the LM level. In methylene blue-stained thick sections, the cytoplasm was light blue and featureless and contained zero to two granules. In TEM sections, from two to ten mitochondria were present as well as abundant rough endoplasmic reticulum (RER) and up to two homogeneous, electron dense cytoplasmic granules (0.5 μm diameter). In addition to these larger granules, up to five smaller granules (~0.15–0.20 μm diameter), which appeared to be coalescing, could be found upon ultramicroscopic examination. The nucleus contained mostly euchromatin with a narrow border of heterochromatin. In Martin et al.’s (1987) classification of shrimp hematopoietic cells, these were termed stromal cells. We now consider them to be the earliest granulocyte stem cells based on the transition of granules in the cytoplasm which presumably form a continuum with small granule stem cells (described in the following paragraph) and with small granule hemocytes.
Table 1

Diameters of shrimp stem cells from hematopoietic tissue and hemocytes from the lumens of hematopoietic vessels

<table>
<thead>
<tr>
<th>Molt stage</th>
<th>C</th>
<th>D₀</th>
<th>D₁₋₂</th>
<th>D₃₋₄</th>
<th>A₁</th>
<th>A₂</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granulocyte</td>
<td>9.8 ± 0.2</td>
<td>9.9 ± 1.8</td>
<td>8.9 ± 1.9</td>
<td>7.1 ± 1.7</td>
<td>7.9 ± 1.8</td>
<td>7.0 ± 1.7</td>
<td>7.4 ± 1.8</td>
<td>9.8 ± 2.0</td>
</tr>
<tr>
<td>Small granule</td>
<td>9.5 ± 1.6</td>
<td>9.3 ± 1.8</td>
<td>9.0 ± 2.1</td>
<td>9.0 ± 1.5</td>
<td>8.7 ± 1.8</td>
<td>8.9 ± 1.9</td>
<td>8.3 ± 1.8</td>
<td>9.5 ± 1.6</td>
</tr>
<tr>
<td>Hyaline</td>
<td>8.6 ± 1.4</td>
<td>7.9 ± 1.4</td>
<td>7.6 ± 1.5</td>
<td>7.6 ± 1.9</td>
<td>7.1 ± 1.6</td>
<td>7.0 ± 1.7</td>
<td>7.1 ± 1.5</td>
<td>8.6 ± 1.4</td>
</tr>
<tr>
<td>Hemocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small granule</td>
<td>7.6 ± 1.3</td>
<td>8.0 ± 0.9</td>
<td>8.0 ± 0.8</td>
<td>6.5 ± 0.7</td>
<td>8.2 ± 0.8</td>
<td>8.2 ± 1.0</td>
<td>7.9 ± 1.6</td>
<td>7.6 ± 1.3</td>
</tr>
<tr>
<td>Hyaline</td>
<td>6.6 ± 0.3</td>
<td>6.7 ± 0.8</td>
<td>6.6 ± 0.8</td>
<td>5.5 ± 0.8</td>
<td>5.8 ± 0.7</td>
<td>6.8 ± 0.7</td>
<td>6.7 ± 1.0</td>
<td>6.6 ± 0.3</td>
</tr>
</tbody>
</table>

* Denotes values which are statistically different from values of preceding molt stage, t-test, P < 0.05.

X ± SD (n) in μm.

Small granule stem cells were distinguished by the presence of many (3–10), small (0.33 μm diameter), ovoid granules in the cytoplasm. These stem cells were usually larger (from 8.3 to 9.5 μm in largest diameter) than the early granulocyte stem cells at identical molt stages (Table 1), and their outlines were irregular (Fig. 1b). The N:C ratio (49.0% ± 8.3%, n = 10) was significantly less than that of the early granulocyte stem cell. The nucleus had a variable shape, ranging from a smooth oval to indented or irregular. One or two nucleoli were present and they were smaller than those of the early granulocyte stem cells. The chromatine pattern was also different from that of the early granulocyte stem cell with a thicker marginal chromatin band and heterochromatin bands radiating from the one or two central nucleoli. The cytoplasm was light blue and featureless at the LM level except for the small homogeneous granules which were often a lighter blue than those of small granule hemocytes. In TEM preparations, the granules appeared to be newly formed by the coalescence of smaller vesicles. Numerous mitochondria and free ribosomes were present.

Small granule hemocytes were followed from various stages of maturation into large granule hemocytes; the transition was accompanied by progressively lower N:C ratios and by increasing numbers and sizes of the cytoplasmic granules. The youngest small granule hemocytes had >10 cytoplasmic granules with diameters of approximately 0.35 μm. Mature small granule hemocytes (Fig. 1c) closely resembled those within the peripheral circulation with round to oval cell contours and >15 0.4 μm diameter granules. However, their mean N:C ratio of 42.2% (SD = 9.6%, n = 10) was higher than that of circulating small granule hemocytes (Martin and Hose, in press). The small granule hemocytes could be distinguished from hyaline cells by their small (6 × 3 μm), ovoid nuclei containing a single, dense, central nucleolus. The marginal chromatin was thicker than those found in granulocyte stem cells, and frequently appeared as a continuous ring or in regularly spaced clumps. Initially, these cells were described as small granule hemocytes lacking cytoplasmic deposits (Martin et al., 1987). They were later identified as small granule granulocytes (Hose and Martin, 1989; Hose et al., 1990). Large granule hemocytes (Fig. 1d) were similar to their circulating counterparts (Martin and Hose, in press) and were distinguished from small granule hemocytes by very low N:C ratios (22.2% ± 5.1%, n = 5) and numerous large (0.8 μm) and smaller cytoplasmic granules.

Hyaline stem cells (Fig. 1e) were the smallest type of stem cell (7.0–8.6 μm diameter, Table 1) with a mean N:C ratio of 45.9% (SD = 8.3%, n = 10). The nucleus was usually oval to oblong, sometimes indented but never angular. The chromatine was denser than those found in granulocyte stem cells. Nucleoli were seldom discernible, even using TEM. A thin rim of dark blue cytoplasm encircling the nucleus conformed to the nuclear contours. Cytoplasmic granules were rarely visible at the LM level. TEM examination revealed a distinctive nuclear characteristic; the presence of thick heterochromatin bands spanning the nucleus. Very little euchromatin was observed. The cytoplasm was filled with tiny spherical (50 nm diameter), electron dense deposits which frequently formed larger clumps measuring up to 186 nm. These deposits were identical to those previously described from a subgroup of granulated hematopoietic cells (Martin et al., 1987) and from circulating hyaline hemocytes (Omori et al., 1989) of the shrimp. The stem cells possessed a Golgi apparatus and numerous mitochondria. Hyaline stem cells lacking cytoplasmic granules were common; these were termed agranular hemocytes in our previous study (Martin et al., 1987). These stem cells sometimes either contained a few small (0.25 μm diameter), homogeneous electron dense granules or their presumptive precursors which were three times larger, having a granular substructure and containing the electron dense core. Up to seven granules with a striated substructure (0.4 μm
Hyaline hemocytes (Fig. 1f) ranged in appearance as a
continuum from the hyaline stem cells to mature cells
indistinguishable from those within the peripheral cir-
culation. As these cells matured, the mean length dimin-
ished to between 5.5 and 6.8 μm (Table I) and the N:C
ratio increased (61.6% ± 6.2%, n = 10). More hetero-
chromatin was present, often as a dense marginal band
in addition to the thick transverse bands present in hyaline
stem cells. In methylene blue-stained LM sections, the
cytoplasm of hyaline cells frequently appeared featureless,
staining metachromatic or intensely basophilic. However,
at the EM level the cytoplasm contained large clumps of
electron dense deposits, striated granules, a few homo-
geneous electron dense granules, and several mitochon-
dria.

**Hemocyte production**

*Mitotic index.* Mitotic activity within the hematopoietic
nodules was cyclical, with the lowest levels bracketing the
cydial interval (stages D3-4 through B) and highest during
intermolt and early pre-molt (stages C–D1-2) (Table II).
Mitotic activity during stage C was about 2.2% and nearly
doubled to a maximum of 4.2% at stage D0. The mitotic
index progressively decreased thereafter until stage A1,
when it reached its lowest level of 0.2%. A secondary burst
of mitotic activity occurred during stage A2 in which 2.0% of
the cells were dividing. In stage B, the mitotic rate de-
declined to 0.8% and then increased again to the stage C
level.

Most of the dividing cells were granulocyte stem cells
and hyaline stem cells (Fig. 2, Table II). Two-thirds of
dividing hyaline cells were stem cells; the remainder were
hyaline hemocytes. Division of small granule hemocytes
was rarely observed, as was division of hemocytes within
the lumens of the hematopoietic tubules. Mitotic rates of
the granulocyte stem cells contributed the bulk of the total
mitotic activity and were responsible for producing the
observed cyclic pattern. In contrast, the mitotic index of
hyaline stem cells remained essentially constant through-
out the molt cycle, exhibiting a modest increase from stage
A1 to D3-4. Like that of granulocytes, the mitotic activity
of hyaline cells was lowest during stage A1, with a subse-
quent pulse during stage A2, then a decrease during
stage B.

**Tubule differential counts.** A molt cycle-related trend
was observed in which the number of cells in the hema-
topoietic tubules was least during stage C and had almost
tripled by stage B (Table III). This observation, along with
the cyclic hemocyte release ending in stage B (see following
section), makes stage C the most convenient portion of
the molt cycle to begin this description of hemocyte
production.

During stage C, a mean of 46 stem cells was present
per cross section of tubule (Table III). A maximum value
of 111 stem cells was recorded in stage D3-4, which
declined to 89 in stage B. Of the stage C stem cells, 80%
were stem cells in the granulocyte line and 20% were hyal-
line stem cells. The proportions of granulocyte stem cells
remained high until stage A1, when hyaline stem cells
comprised about 40% of the total. During stages A2 and
B, the proportion of hyaline stem cells increased to around
80%. Diameters for all stem cell types were greatest in
stage C, then decreased throughout the cycle by 1 to 2
μm (Table I). The smallest diameters were most frequently
recorded in stage B tubules.

Very few mature hemocytes were observed in the tubule
wall from stage C until just before ecdisis (stage D3-4),
with means ranging from 12 to 18 cells (Table III).

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**Table II**

<table>
<thead>
<tr>
<th>Molt stage</th>
<th>C</th>
<th>D0</th>
<th>D1-2</th>
<th>D3-4</th>
<th>A1</th>
<th>A2</th>
<th>B</th>
<th>C</th>
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<td><strong>Hyaline cells</strong></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Stem cells</td>
<td>0.27</td>
<td>0.42</td>
<td>0.43</td>
<td>0.54</td>
<td>0.11</td>
<td>0.36</td>
<td>0.09</td>
<td>0.27</td>
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<tr>
<td>Hemocytes</td>
<td>0.13</td>
<td>0.08</td>
<td>0.08</td>
<td>0.04</td>
<td>0.02</td>
<td>0.16</td>
<td>0.05</td>
<td>0.13</td>
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<tr>
<td>Total hyaline</td>
<td>0.40</td>
<td>0.50</td>
<td>0.51</td>
<td>0.58</td>
<td>0.13</td>
<td>0.52</td>
<td>0.14</td>
<td>0.40</td>
</tr>
<tr>
<td><strong>Granulocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stem cells</td>
<td>1.68</td>
<td>3.53</td>
<td>1.42</td>
<td>0.51</td>
<td>0.12</td>
<td>1.34</td>
<td>0.60</td>
<td>1.68</td>
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<tr>
<td>Small granule hemocytes</td>
<td>0.09</td>
<td>0.17</td>
<td>0.04</td>
<td>0.02</td>
<td>0.00</td>
<td>0.12</td>
<td>0.06</td>
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<tr>
<td>Total granulocyte</td>
<td>1.77</td>
<td>3.70</td>
<td>1.46</td>
<td>0.53</td>
<td>0.12</td>
<td>1.46</td>
<td>0.66</td>
<td>1.77</td>
</tr>
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</table>

**Total mitotic activity**  
2.17 ± 0.40*  4.20 ± 0.89  1.97 ± 0.49  1.11 ± 0.33*  0.25 ± 0.18*  1.98 ± 1.12  0.80 ± 0.27*  2.17 ± 0.40*

* Denotes values significantly different from molt stage D0, analysis of variance and Student-Newman-Keuls, P = 0.05.  
Values for total mitotic activity are the mean and standard error for five individuals.
Figure 2. Transmission electron micrographs and light micrographs (inserts) of dividing cells. Spindle microtubules and centrioles are not visible in the electron micrographs, but both cells lack nuclear envelopes. (A) Granulocyte stem cell in metaphase; insert shows a cell in anaphase (arrows = granules). (B) Hyaline cell in prophase or metaphase. Right insert shows a cell in telophase and left insert same cell at a higher magnification (17,600×) of a typical granule with a striated substructure from the hyaline cell. Granulocyte and hyaline stem cells may be distinguished by differences in the electron density of the cytoplasm (note the darker cytoplasm of the hyaline cell) and the substructure of the cytoplasmic granules (arrows). Note the homogeneous substructure of the electron dense granules in the granulocyte stem cell. Electron micrographs 9,600×; scale bar = 5 μm. Light micrographs—methylene blue, 1,800×.

Oocytes constituted only 12–20% of the total hematopoietic cells during these stages. After molting, the number of hemocytes almost tripled to 42. Throughout the post-ecdysial stages (A, to B), their numbers doubled as maturing hemocytes accumulated within the tubules. In stage B, 48% of the hematopoietic cells were hemocytes.

Cells of the granulocyte line predominated within the intermolt and early pre molt hematopoietic tissue (stages C through D₁₋₂). Mitotic activity progressively increased from stage A₁ to a peak at stage D₀. Numbers of granulocyte stem cells were lowest during stage C, and increased to a maximum during stage D₃₋₄ (Fig. 3). During stages B and C, granulocyte stem cells were usually the earliest forms with zero or only a few coalescing granules and a distinctive large central nucleolus. Stem cells in stage C tubes were the largest of any molt stage, averaging 9.8 μm in diameter (Table I). More mature, smaller stem cells were typical of stage D tubes; these contained numerous electron dense granules. Small granule hemocytes were the most prominent hemocyte type during stages D₀₋₂, after which they declined in abundance (Fig. 4). Large granule hemocytes first appeared in stage C tubes and, although they became more abundant until stage D₁₋₂, they were infrequently observed. Despite the high mitotic rate of granulocytes (Fig. 3a) and the increasing numbers of stem cells in the granulocyte line during stages C

Table III

<table>
<thead>
<tr>
<th>Molt stage</th>
<th>C</th>
<th>D₀</th>
<th>D₁₋₂</th>
<th>D₃₋₄</th>
<th>A₁</th>
<th>A₂</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemocytes</td>
<td>12.0±3.9</td>
<td>11.0±1.4</td>
<td>18.4±5.2</td>
<td>14.8±3.2</td>
<td>41.8±19.9</td>
<td>38.4±9.9</td>
<td>83.0±17.7</td>
<td>12.0±3.9</td>
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<tr>
<td>Stem cells</td>
<td>46.2±3.0</td>
<td>60.8±11.1</td>
<td>97.0±9.7</td>
<td>111.4±8.6</td>
<td>97.2±18.5</td>
<td>90.8±23.2</td>
<td>88.6±11.5</td>
<td>46.2±3.0</td>
</tr>
<tr>
<td>Total cells</td>
<td>58.2±4.3</td>
<td>71.8±11.9</td>
<td>115.4±6.1</td>
<td>126.0±11.7</td>
<td>139.0±29.7</td>
<td>129.2±26.5</td>
<td>171.6±27.5</td>
<td>58.2±4.3</td>
</tr>
</tbody>
</table>

* Denotes total cell counts significantly different from those of molt stage B, analysis of variance and Student-Newman-Keuls, P = 0.05. Values are mean and standard error from five individuals.
Figure 3. Mitotic index of granulocytes (% of cells in mitosis) (▲) and numbers of granulocyte stem cells (■) in the hematopoietic tissue of shrimp throughout the molt cycle. Error bars are ±1 standard deviation. Numbers of granulocyte stem cells were significantly higher during molt stages D1-4.

through D3-4 (Fig. 3b), surprisingly few maturing granulocytes were observed within the wall of the hematopoietic tubules (Fig. 4a). They were apparently released soon after production (see next section). A secondary, post-ecdysial burst of granulocyte production reflected the enhanced mitotic activity of stage A2. Hemocytes produced during stages A2 through B accumulated within the tubules. The fluctuating numbers of hemocytes within the tubules reflect differences in release rates of maturing granulocytes and will be discussed in the section on hemocyte release.

In contrast to granulocyte production, which occurred primarily in stages C through D1-2, hyaline hemocytes were produced at comparatively low rates during these stages. Most hyaline hemocytes were instead produced during the stages bracketing ecdysis (D3-4 through A). Mitotic activity in the hyaline stem cells increased from a low level during stage B to a maximum during stage D3-4 (Fig. 5). The numbers of hyaline stem cells and hyaline hemocytes within the tubules peaked shortly thereafter, in stage A1 (Fig. 6). Although numbers of hyaline stem cells then decreased to reach their lowest level at stage C, numbers of hyaline hemocytes remained elevated throughout stage B, apparently aided by a pulse of hyaline stem cell division during stage A2. Hyaline stem cell sizes were greatest during molt stage C, with a mean diameter of 8.6 μm (Table 1). They then declined to 7.6 μm during stage D3-4 and attained their minimum size of approximately 7.1 μm from stage A1 through stage B.

Hemocyte maturation. Observations of cells within hematopoietic tubules suggest that granulocytes and hyaline hemocytes progress along two separate lines of maturation. Because stage C tubules contained the earliest granulocyte stem cells recognizable at the LM level, electron micrographs of tubules from the previous stage (B) were examined for cells that might be precursors to the granulocyte stem cell or, possibly, a common progenitor of both hemocyte lines. Stem cells in stage B tubules were of two types—those that possessed coalescing cytoplasmic granules and that lacked the deposits (granulocyte stem cells) and those which contained the distinctive cytoplasmic deposits of the hyaline cell line. No stem cell could be found that possessed ultrastructural features common to both lines of hemocytes.

Granulocytes arose from an undifferentiated cell type typical of stem cells with its large size, a nucleus containing predominantly euchromatin, and a high N:C ratio. Its cytoplasm lacked specialized organelles. This cell was common in stage A2 and B specimens (Fig. 1a). As maturation of granulocyte stem cells progressed, cells developed a few small granules which were in various stages of coalescence (Fig. 1b); these were frequently observed in stage B tubules (Fig. 7). Although they occasionally appeared larger than the undifferentiated forms, maturing stem cells had a progressively lower N:C ratio and condensed electron dense granules were present. As granulocyte stem cells matured into small granule hemocytes and then into large granule hemocytes, the number of cytoplasmic granules increased, the granules became larger, and the N:C ratio further decreased (Fig. 1c, d). Clusters of granulocytes in various stages of maturation were present in stage C and D0 tubules; they frequently contained one to several granulocyte stem cells and small granule stem cells as well as small granule and occasional large granule hemocytes (Fig. 8). The clusters sometimes appeared to be bounded by a thin basal lamina.

Hyaline stem cells resembled undifferentiated granulocyte stem cells except for their lower N:C ratio, the lack of a prominent central nucleolus, the predominant heterochromatin within the nucleus, and the more electron-dense cytoplasm. Early hyaline stem cells lacking cytoplasmic granules were most readily observed in stage C tubules. As the hyaline stem cell matured, it became smaller (Table 1), the cytoplasmic deposits organized into large, dense clumps, and the nucleus became more condensed. In these stem cells, abundant in stage D tubules, the cytoplasm also contained coalescing granules with an obvious striated substructure as well as a few small, homogeneous electron dense granules. Hyaline hemocytes were smaller than the stem cells (Table 1) and the cytoplasmic granules resembled those present in their circulating counterparts. Clusters of maturing hyaline stem cells and hemocytes usually contained fewer cells than did the granulocyte clusters; they were most readily observed in late stage D tubules (Fig. 9). Like the granulocyte clusters, hyaline clusters occasionally appeared to be enclosed by a basal lamina.

Hemocyte release

Lumen counts. Release of hyaline cells and granulocytes into the lumen of the hematopoietic tubules occurred at
Figure 4. Numbers of small (■) and large (▲) granule hemocytes within shrimp hematopoietic tubules (A) and the lumens of the tubules (B) throughout the molt cycle. Mean of five shrimp, four tubules/shrimp. Error bars are ±1 standard deviation. Significantly more small granule hemocytes were present in the tubules during molt stage B, probably reflecting increased mitotic activity during stage A₂. The only significant difference in the numbers of small granule hemocytes released into the lumen was an elevation during stage D₂-2 corresponding to the surge in mitotic activity during stage D₁. Mean tubule diameters remained similar throughout the molt cycle, so cell counts could be directly compared.
different times during the molt cycle. Granulocytes appeared to be released soon after production during intermolt and early D stages (Fig. 4). However, a second, smaller pulse of granulocyte production occurred during stage A2 with some of the cells apparently released during this stage and the rest accumulated in the tubules during stage B and released during stage C. Characteristics of the released cells supported these inferences. Sizes of released granulocytes during stages D0-D2 were large (Table I) and they more closely resembled small granule stem cells (i.e., fewer granules and a higher N:C ratio than most circulating small granule hemocytes), suggesting that they were indeed released soon after production. Similarly, small granule cells released during stage A were larger and more immature compared to those released during B and C, which were smaller and contained more granules.

Production of hyaline hemocytes peaked during the ecdysial period (stage D3-A through A). Hyaline cells appeared to be released soon after their production during stages C through D1-C. Many of these cells appeared to be maturing hyaline stem cells since their size approximated that of the stem cells (Table I), the N:C ratio was lower than that of circulating hyaline hemocytes, and the cytoplasm was frequently agranular. During stages D3-A1 and A1, however, the size of released cells significantly declined, and they appeared more like circulating hyaline hemocytes with heavily condensed chromatin and clumped cytoplasmic deposits, suggesting that further maturation occurred while they accumulated in the tubules for post-ecdysial release. There was a second pulse of hyaline hemocyte production during stage A2 with the apparent rapid release of these relatively large, immature hyaline cells occurring during stages A2 and B.

Occasionally, dividing cells were present inside the lumen of the hematopoietic tubules. These were most frequently cells intermediate between small granule stem cells and hemocytes, in addition to a few large, immature hyaline hemocytes. No relationship to molt stage could be detected due to the rarity of mitotic cells in the lumen.

Release through endothelium. Most hemocytes exited the tubules through the endothelium. During stages C and early D, diapedesis of individual hemocytes (usually granulocytes) was most common, with the endothelial barrier remaining intact (Fig. 10a). Around the ecdysial period, however, endothelial channels were observed through which groups of maturing hemocytes, usually hyaline hemocytes, were released into the lumen (Fig. 10b). By stage B, the endothelial lining was difficult to distinguish since it appeared to be composed of isolated cells detached from the underlying stroma and interspersed with groups of exiting hemocytes.

Regeneration of the endothelium began during stages C and D0, as evidenced by the presence of cells undergoing mitosis, thickening of the endothelial cells, and the continuous appearance of the basal lamina. The regenerated endothelium seen in pre-ecdysial tubules appeared continuous save for isolated diapedesis by individual hemocytes. However, during stage D3-A, groups of emigrating hemocytes formed channels between individual endothelial cells. The endothelial barrier was progressively destroyed by the hyaline hemocytes released during the ecdysial interval. By stage A2, the endothelial cells appeared attenuated and were frequently detached from the underlying collagenous stroma. In stage B, large channels separating the endothelial cells were visible in electron micrographs (Fig. 11), and some of the endothelial cell nuclei appeared to be degenerating. Also in stage B, many circular formations resembling myelin figures were dispersed through the collagenous stroma. We could not identify any cell type involved in production or repair of the collagenous matrix forming the stroma of the hematopoietic tubules.

Release through capsule. A small percentage (<5%) of hemocytes was released via migration through the outer capsule of the tubule into the adjacent hemal space. In each tubule, only one or two hemocytes were usually present in the capsular network of collagen fibrils. This percentage did not change considerably throughout the molt cycle except for stage D3-A in which 12% of the hemocytes were observed to be migrating through the capsule. There were no obvious differences between the categories of hemocytes exiting the capsule and those released via the endothelium.

Discussion

Our study shows that changes in hematopoietic activity of the shrimp *Sicyonia ingentis* are related to the molt cycle. Hemocyte production and release correspond with the known physiological roles of hemocytes. Hyaline hemocytes initiate hemolymph coagulation (Omori et al.,
Figure 6. Numbers of hyaline hemocytes within shrimp hematopoietic tubules (A) and the lumens of the tubules (B) throughout the molt cycle. Mean of five shrimp, four tubules/shrimp. Error bars are ±1 standard deviation. The number of hyaline hemocytes inside the tubules was significantly elevated during molt stages A₁-B (post-ecdysis), concurrent and following the increased number of hyaline stem cells during stage A₁ as shown in Figure 5. Although a similar pattern of hyaline hemocyte release was observed during stages A₁-B, differences between the numbers of hyaline hemocytes inside the lumen were not significant due to high variability among the counts. Mean tubule diameters remained similar throughout the molt cycle, so cell counts could be directly compared.
Figure 7. Light micrograph of stage A₂ tubule containing mostly stem cells. Hemocytes appeared to have been already released from this tubule. Contrast the relative acellularity here with the more abundant hemocytes and large stem cells present in Figures 8 and 9. L = lumen. Methylene blue. 1430×; scale bar = 20 μm.
1989) and contribute to formation of the pre-ecdysial procuticle and sclerotinization of the endocuticle at postecdysis (Vacca and Fingerman, 1983); they are thus essential throughout the molt cycle but also figure importantly during ecdysis. Hyaline hemocytes are produced at fairly constant levels from early intermolt (stage C) until immediately before molting (stage D3-4). Some release occurs throughout this period, but many accumulate within the HPN until a large burst is released at postecdysis (stages A–B). Elevated numbers of hyaline hemocytes have previously been reported around ecdysis in the hemolymph (Kollman, 1908). Despite the use of slightly different molt stages in their analysis, Tsing et al. (1989) reported a similar pattern for their “lysing hemocytes,” which undoubtedly are hyaline hemocytes. Not only have elevated numbers of circulating hyaline cells been noted around the time of ecdysis, but they have been observed migrating into the epidermis (Vacca and Fingerman, 1983). The latter investigators found that cross-linking of the endocuticle protein matrix is accomplished by diphenoic metabolites apparently synthesized or carried by the hyaline hemocyte. They further speculated that during molting, lysis of hyaline hemocytes liberates diphenoics into the hemolymph which are translocated into the soft cuticle. We have also observed the accumulation of hyaline hemocytes beneath the forming cuticle but have been unable to duplicate Vacca and Fingerman’s (1983) demonstration of diphenoic intermediates within these cells.

Granulocytes are primarily responsible for defense against foreign particles (Söderhäll et al., 1988; Hose and Martin, 1989). Studies combining ultrastructural and cytochemical cell identification demonstrate that small granule hemocytes phagocyte small particles like bacteria (Hose et al., 1990), although another group claims that hyaline hemocytes are also phagocytic, albeit at extremely low rates (Söderhäll et al., 1988). In contrast, the role of large granule hemocytes is undisputed in the encapsulation of large foreign bodies such as metazoan parasites (Hose et al., 1990). Although other cellular components may be involved, the prophenoloxidase system located in the granules and secretory vesicles of large granule hemocytes is considered to be the primary defense against most foreign molecules (Söderhäll et al., 1986). Granulocytes, therefore, are essential to the host throughout the molt cycle. Small granule hemocytes are produced at the highest rate and released during intermolt and early premolt. Maturation of small granule hemocytes into large granule hemocytes occurs both in the hematopoietic tissue as well as in the peripheral circulation. This would ensure the constant production of large granule hemocytes, which eventually must become senescent and apparently lose the phagocytic portion of their defensive ability (Hose and Martin, 1989). The present study demonstrated that a minor pulse of granulocytes is produced and liberated during the postecdysial stage A2 although available studies indicate that the duration of this stage is only a few hours (Skinner, 1962; Anderson, 1985). Similar results were reported by Tsing et al. (1989), where total granulocyte counts increased in the hemolymph from stage C to a maximum at stage D3-4, followed by a large decline during the period immediately prior to ecdysis (stage D4). A secondary increase was observed during stages A–B, followed by a decrease during stage B2. Granulocytes may also participate in hardening of the exoskeleton. According to Vacca and Fingerman (1983), granulocytes cooperate in producing the procuticle and hardening the endocuticle by contributing formative proteins to be cross-linked by diphenoics released by hyaline hemocytes. Granulocytes predominated in the cuticle prior to ecdysis and appeared to be degranulated by postecdysis. They speculated that release of granules into the hemolymph liberated proteins which may be involved in tanning, basement membrane formation, and wound healing. Other studies have suggested that granulocytes may activate an alternate pathway of hemolymph coagulation (Ravindranath, 1980; Durliat, 1985; Söderhäll et al., 1988). Certainly the abundance of granulocytes as well as hyaline cells to repair the exoskeleton and fight infection is advantageous during ecdysis when the newly formed exoskeleton is soft and most susceptible to damage.

The only other study providing morphologic information on hematopoiesis throughout the molt cycle is Johnson’s (1980) description of the hematopoietic tissue in the blue crab Callinectes sapidus. These photomicro-

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**Figure 8.** Light micrograph of stage D3 tubule showing granulocyte cluster (arrows). Methylene blue. 1700×; scale bar = 12 µm.

**Figure 9.** Light micrograph of stage D1-2 tubule showing a hyaline cell cluster (arrows). Methylene blue. 1700×; scale bar = 12 µm.

**Figure 10.** Light micrographs of hemocytes exiting from the wall of the hematopoietic tubule into the lumen (L). (A) Diapedesis of three hemocytes through the intact endothelium (E) of an intermolt shrimp. (B) DURING MOLTING, THE ENDOTHELIAL BARRIER IS DISCONTINUOUS; A SINGLE ENDOTHELIAL CELL (E) IS PRESENT AT THE TOP. A large group of hemocytes enters the lumen through a channel in the endothelium. Methylene blue. 1066×; scale bar = 15 µm.

**Figure 11.** Transmission electron micrograph of subendothelial spaces (*) and myelin figures (arrows) in the stroma of a stage B tubule. 5250×; scale bar = 8 µm.
graphs show hemocytes resembling granulocytes and corresponding stem cells filling the intermolt tissues. Most of the stem cells had the prominent cytoplasmic granules and nuclear characteristics (large central nucleolus) typical of the granulocyte line. These cells appear similar to the granulocyte stem cells described here in the shrimp and also are most evident in stages B through D devices 2. In the crab, granulocytes produced during intermolt apparently were released soon after production since no increase in intermolt tissue thickness was noted despite the enhanced mitotic activity occurring from stages B onwards to late C (Johnson, 1980). Pre- and post-molt hematopoietic tissue appeared to be filled with smaller (5-6 μm diameter) hemocytes with a high N:C ratio, which may be hyaline stem cells and hemocytes. Johnson described a second type of stem cell with densely basophilic, homogeneous nuclei which lack distinct nucleoli. These characteristics are typical of the hyaline stem cells described in the shrimp which predominate in the pre- and post-molt hematopoietic tissue.

In both Johnson's (1980) study and ours, some individual variability was noted in the types of hemocytes produced, (2) in the mitotic indices, and (3) between different portions of the hematopoietic tissue. Individual variability would be expected to result from differences in health status and environmental conditions. For example, increased production of granulocytes should occur in response to infectious disease. Studies have documented enhanced hematopoiesis in decapods infected with a virus (Lightner et al., 1983) and a fungus (Hose et al., 1984), although they did not categorize the types of hemocytes produced. Johnson (1980) noted the presence of mature granulocytes in the hematopoietic tissue of blue crabs infected with certain viruses, bacteria, and protozoa. However, stimulation of hematopoiesis may only be a transient response to an overwhelming acute infection, as has been shown for the fungus Fusarium solani. In early stages of the disease, mitosis is enhanced, but in advanced cases, the circulating hemocyte count drops and the hematopoietic tissue becomes necrotic (Hose et al., 1984). Investigations are underway in our laboratory to quantify mitotic rates and hemocyte production in bacteria-infected shrimp.

Environmental stress can also modulate hematopoiesis. For instance, mitotic activity is greatly reduced in overwintering blue crabs, and degenerating hematopoietic cells are common (Johnson, 1980). Developing granulocytes routinely occur in the hematopoietic tissue only at this time. Hemocyte production becomes enhanced as spring water temperatures increase. Long-term laboratory maintenance of blue crabs resulted in a similar increase in mitotic activity. Other factors with virtually undescribed effects on hematopoiesis include reproductive status, diet (Bauchau and Plaquet, 1973), and environmental pollution.

Although the observations of hemocyte maturation described here support our previous theory that the hyaline hemocytes and granulocytes comprise two separate lines (Martin et al., 1987; Hose et al., 1990), the scheme may be incomplete. Is there a common pluripotential stem cell which gives rise to the hyaline and granulocyte stem cells, and if so, where is it located? We were unable to find any cells within the hematopoietic tubules which possessed shared characteristics, such as the presence of striated granules or cytoplasmic deposits in a cell with numerous homogeneous granules, a large central nucleolus, or a nucleus containing predominately euchromatin. Such a cell might be detected in vivo autoradiography experiments or in long-term in vitro maintenance of hematopoietic tissue using the culture methods described by Brody and Chang (1989). However, in their study, no mitosis was observed in contrast to earlier experiments where mitosis continued through the 12-day culture period (Fischer-Piette, 1931). Advances or modification of culture techniques and appropriate selection of mitotically active molt stages may yield sufficient material to follow the maturation of individual cells.

Any understanding of decapod hematopoiesis must recognize its interdependence upon a multiplicity of physiological and pathological states. Knowledge of the influences of normal physiology and disease status are essential before hematopoiesis can be effectively modulated to combat infectious disease or alleviate health problems arising in mariculture situations.

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