# Characterization of Hemocytes of an Estuarine Gastropod Mollusc, *Clithon retropictus*, Based on Lysosomal Enzymes

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*Clithon retropictus* is an estuarine neritid gastropod mollusc which can preserve high levels of thermostable direct hemolysin-producing strains of *Vibrio parahaemolyticus* in the alimentary tract [8, 10]. From the fact that small individuals were predominant in *C. retropictus* community at the stations of an estuary where *V. parahaemolyticus* was detected at high levels [7, 8, 11], juvenile *C. retropictus* was suspected to be more important for the reservoir of *V. parahaemolyticus* than adult one.

*V. parahaemolyticus* was observed to be eliminated from a marine neritid gastropod, *Nerita albicilla*, within 3 days [10]. Hemocytes from *C. retropictus* were less active than those from *N. albicilla* in migratory response to *V. parahaemolyticus* [9] and in attachment of *V. parahaemolyticus* [12]. Most hemocytes of adult *C. retropictus* and *N. albicilla* were found to be amoebocytes with pseudopodia and granules [14]. As lysosomal enzymes of hemocytes are known to play a major role in the defense mechanism of some gastropods [2, 16], lysosomal enzymes in hemocytes of adult *C. retropictus* were expected to be more active than those of juvenile *C. retropictus* and less active than those of *N. albicilla*. This study describes the distribution of three lysosomal enzymes within populations of circulating hemocytes from these gastropods.

Juvenile and adult specimens, 0.5–1.0 g and 2.0–3.5 g weight respectively, of *C. retropictus* and *N. albicilla* were maintained in UV-irradiated recirculating artificial seawater with salinities of 15 permil (‰) for *C. retropictus* and 35‰ for *N. albicilla* as described previously [10]. The opercula of the gastropods were pushed in to allow hemolymphs to leak out from apertures of the animals. Drops of the hemolymphs were placed on poly-L-lysine-coated glass coverslips and incubated at 25°C for 10 min in a moist chamber. The hemocytes were then fixed with 4% cold paraformaldehyde (pH 7.4) for 5 min and maintained in Holt's gum sucrose. The hemocytes were stained by the $\alpha$-naphtyl acetate method [14] for non-specific esterase, by the naphthol AS-BI phosphate method [1] for acid phosphatase and by the naphthol AS-BI $\beta$-glucuronidase method [5] for $\beta$-glucuronidase and observed under light microscopy. Mouse peritoneal macrophages were stained simultaneously with the molluscan hemocytes as positive control preparations.

Distributions of three lysosomal enzymes in hemocyte populations of *C. retropictus* and *N. albicilla* observed by the staining reactions are summarized in Table 1. In adult specimens, most hemocytes from two gastropods were positive for non-specific esterase while *C. retropictus* hemocytes strongly stained for the esterase (13.9%) were significantly lower in rate than *N. albicilla* hemocytes (34.3%) ($p<0.001$). Acid phosphatase was stained strongly in 34.8% of *N. albicilla* hemocytes and weakly in 17.5% of *N. albicilla* and *C. retropictus* hemocytes. Beta-glucuronidase was stained weakly in approximately 10% of hemocytes from two gastropods. More than 92% of mouse macrophages were stained strongly in these enzymes.

Table 1. Activity of three lysosomal enzymes in *C. retropictus* and *N. albicilla* hemocytes as determined by staining reaction

| Enzyme | Number of hemocytes[a)] (%) | | | | |
|---|---|---|---|---|---|
| | *C. retropictus* | | *N. albicilla* | | Mouse |
| | Juvenile | Adult | Juvenile | Adult | macrophage |
| Non-specific esterase | | | | | |
| Strongly positive | 11/398( 2.8)** | 55/397(13.9)*** | 78/363(21.5)** | 206/601(34.3) | 177/190(93.1) |
| Weakly positive | 373/398(93.7) | 335/397(84.4) | 280/363(77.1) | 387/601(64.4) | 13/190( 6.9) |
| Negative | 14/398( 3.5) | 7/397( 1.8) | 5/363( 1.4) | 8/601( 1.3) | 0/190( 0 ) |
| Acid phosphatase | | | | | |
| Strongly positive | 0/327( 0 ) | 0/332( 0 ) | 94/412(22.8)** | 177/508(34.8) | 102/110(92.7) |
| Weakly positive | 23/327( 7.0)** | 58/332(17.5) | 111/412(26.9) | 89/508(17.5) | 7/110( 6.4) |
| Negative | 304/327(93.0) | 274/332(82.5) | 207/412(50.2) | 242/508(47.6) | 1/110( 0.9) |
| $\beta$-glucuronidase | | | | | |
| Strongly positive | 0/359( 0 ) | 0/343( 0 ) | 0/325( 0 ) | 0/367( 0 ) | 148/160(92.5) |
| Weakly positive | 19/359( 5.3)* | 34/343( 9.9) | 27/325( 8.3) | 37/367(10.1) | 11/160( 6.9) |
| Negative | 340/359(94.7) | 309/343(90.1) | 298/325(91.7) | 330/367(89.9) | 1/160( 0.6) |

a) Number of hemocytes determined/total number of hemocytes counted. * and **: Significantly lower than adult specimen ($p<0.05$ and $p<0.001$, respectively). ***: Significantly lower than adult *N. albicilla* ($p<0.001$).

In *C. retropictus* hemocytes, most hemocytes from juvenile specimens were positive for non-specific esterase while the hemocytes from juvenile specimens stained strongly for the esterase (2.8%) were significantly lower in rate than those from adult specimens (13.9%) ($p<0.001$). Acid phosphatase-positive and $\beta$-glucuronidase-positive hemocytes from juvenile specimens were significantly lower in rate than those from adult specimens ($p<0.001$ and 0.05, respectively), though these enzymes were stained weakly in any enzyme-positive hemocytes. In *N. albicilla* hemocytes, on the other hand, enzyme-positive hemocytes from juvenile specimens were similar in rate to those from adult specimens in any enzymes though the hemocytes strongly-stained in non-specific esterase and acid phosphatase were significantly lower in rate in juvenile specimens than in adult specimens ($p<0.001$). The molluscan hemocytes showed the same staining patterns for three enzymes. As most of mouse macrophages were stained strongly for three enzymes, the staining technique was reliable. From these evidences, it was concluded that adult *C. retropictus* hemocytes were less active than adult *N. albicilla* hemocytes and more active than juvenile *C. retropictus* hemocytes in lysosomal enzymes.

Lysosomal enzymes have been detected from hemocytes of various molluscs [6, 17, 18]. In *Biomphalaria glabrata*, vector host gastropod of *Schistosoma mansoni*, non-specific esterase-positive or acid phosphatase-positive hemocytes were higher in rate in schistosoma-resistant strain than in schistosoma-susceptible strain [4]. In our previous studies, *C. retropictus*, but not *N. albicilla*, was found to support long-term survival of *V. parahaemolyticus* in the alimentary tract [10]. From these evidences, low activity of hemocytes of *C. retropictus* hemocytes, especially juvenile one, in the lysosomal enzymes seems to be related with the survival of *V. parahaemolyticus* in the gastropod.

Hemocytes from juvenile specimens of *C. retropictus* were less active than those from adult ones while those from juvenile specimens of *N. albicilla* were similar to those from adult ones in the activity of the lysosomal enzymes. *C. retropictus* hemocytes revealed two morphologically different types, round cells with few ruffles and spreading cells with many ruffles, when the molluscan hemolymph was incubated on glass surface at 25°C for 10 min, in which the spreading cells accousnted for 43 and 92% of hemocytes from juvenile and adult specimens, respectively (unpublished data). In *B. glabrata* [3], peroxidase-positive hemocytes from juvenile specimens (20–24%) were reported to be lower in rate than those from adult ones (74–84%). From these evidences, rate of immature hemocytes in the hemocyte population might be higher in juvenile *C. retropictus* than in adult one.

The lysosomal enzymes in *C. retropictus* and *N. albicilla* hemocytes were shown to be less active than those in mouse macrophages. *B. glabrata* hemocytes infected with *Echinostoma paraensei* were reported to have an acid phosphatase activity in a similar level to human polymorphonuclear leukocytes [15]. Lysosomal enzymes in hemocytes of the neritid gastropods might be activated by the infection of some organisms.

There are many evidences on the concentrations of various pathogens to animals inhabiting estuaries and gulf coasts. The concentrations might be related, at least in part, with weak defense systems of the animals. Our previous observations [9, 10, 12] on *C. retropictus* related with *V. parahaemolyticus* seem to be consistent with the hypothesis. The present study would have provided important informations to elucidate the mechanisms for the pathogens to be concentrated in the estuarine gastropods.

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