Fine Structure of Spreading Hemocytes of an Estuarine Gastropod Mollusc, *Clithon retropictus*

Norichika H. KUMAZAWA, Tomohiro IMAGAWA[1)], Takahiko TANIGAWA[2)], and Yoshinori TANAKA[2)]

Department of Veterinary Public Health and [1)]Veterinary Anatomy, Faculty of Agriculture, Tottori University, Tottori 680 and [2)]Department of Bacteriology, Faculty of Medicine, Tottori University, Yonago 683, Japan

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Clithon retropictus, an estuarine neritid gastropod mollusc, is an important reservoir of thermostable direct hemolysin-producing strains of *Vibrio parahaemolyticus* [2, 7]. The organism could survive long in the alimentary tract of *C. retropictus* but was eliminated rapidly from two marine neritids, *Nerita albicilla* and *Heminerita japonica* [3, 5]. As *V. parahaemolyticus* was detected from juvenile *C. retropictus* at higher levels than from adult animal [2, 4, 7], analyses of the defense system of the juvenile animal would be essential to elucidate mechanisms for *C. retropictus* community to preserve a high level of the organism.

Morphology of hemocytes taken from freshwater gastropods has been observed by many researchers [1, 12–14], while no evidence was seen on fine structure of hemocytes of estuarine gastropods until our research works started. Scanning electron micrographs of *C. retropictus* hemocytes showed that circulating hemocytes were composed of round and spreading cells and that juvenile's hemocytes were less in rate of the spreading cells than adult's hemocytes [10], which was consistent with those of two freshwater gastropods, *Lymnaea stagnalis* [1] and *Biomphalaria glabrata* [12]. In addition, hemocytes of juvenile, but not adult, *C. retropictus* required *C. retropictus* plasma to exhibit chemotaxis to *V. parahaemolyticus* strains [8]. Hemocytes of juvenile *C. retropictus* were less active than adult's hemocytes in lysosomal enzymes [9]. From these evidences, circulating hemocytes of juvenile *C. retropictus* have been suggested to be immature in their surface structure and some functions. In the present study, inner structure of juvenile's hemocytes was observed to characterize the morphological bases of the delayed maturation of *C. retropictus* hemocytes.

Hemolymphs taken from adult and juvenile *C. retropictus* weighing 3.5 and 0.8 g, respectively, were mounted on plastic sheets and incubated at 25°C for 30 min to allow hemocytes to adhere to the sheets. After washing in 0.1 M phosphate buffer (pH 7.3) (PBS), hemocytes adhered to the sheets were fixed in 2.5% glutaraldehyde in PBS for 1 hr, washed in PBS, postfixed in 1% osmium tetroxide in the same buffer for 1 hr, dehydrated and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined with Hitachi HU-12A electron microscope at 100 kV.

Thin sections of spreading hemocytes from adult and juvenile *C. retropictus* adhered to plastic sheets are shown in Figs. 1–3. Lysosomes, mitochondria and phagosome

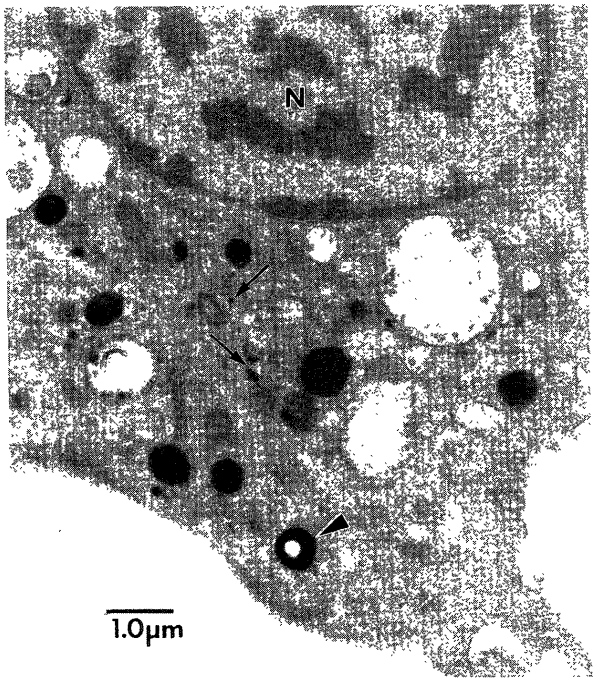

Fig. 1. Spreading hemocytes of adult *C. retropictus*. → lysosome. ▶ lipid particle.

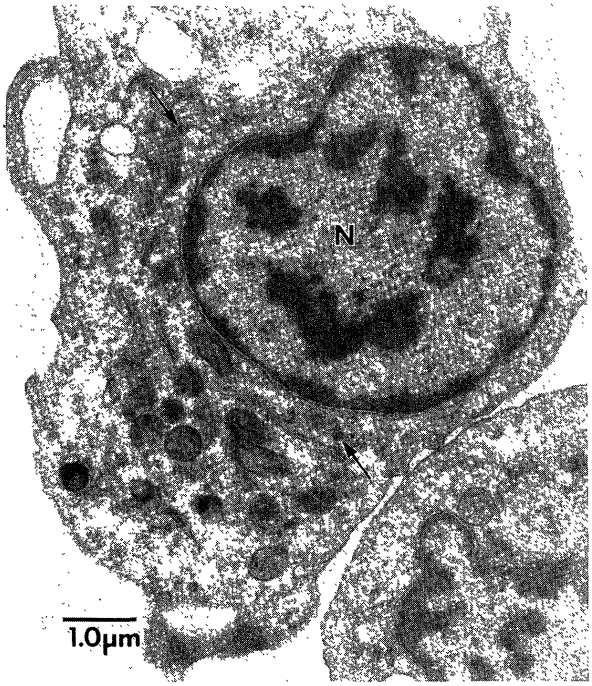

Fig. 2. Spreading hemocytes of juvenile *C. retropictus*. → lysosome.

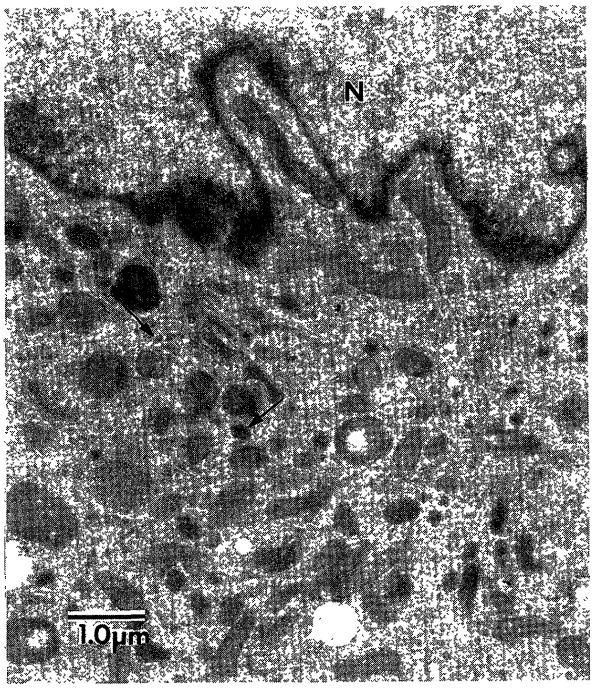

Fig. 3. Spreading hemocytes of juvenile *C. retropictus*. → lysosome.

vesicles were developed well in adult's and juvenile's hemocytes. Lipid particles were seen in the cytoplasm of adult's, but not juvenile's, hemocytes, which was differentiated from the secondary lysosomes in morphological characters of spherical form filled with homogeneous electron-dense materials. Golgi apparatus was less developed in juvenile's hemocytes.

Lipid particles found in adult's, but not juvenile's, hemocytes in the electron micrograph were most characteristic in morphological difference between adult's and juvenile's hemocytes. To confirm for the particles to contain lipid, the hemocytes were stained by Sudan black B and observed under an optical microscope at a magnification of 1,000. Sudan black B-positive particles including secondary lysosomes were counted to a number of 26.2±7.9 per adult's hemocytes which was significantly more than that of 19.7±7.1 per juvenile's hemocytes ($p<0.001$). Lipid particles in adult's hemocytes were larger than those in juvenile's hemocytes in Sudan black B-stained optical micrograph. From these evidences, circulating hemocytes of juvenile *C. retropictus* was suggested to be immature in their inner structure.

Phagosome vesicles were seen in adult's and juvenile's hemocytes. *V. parahaemolyticus* was observed to attach to hemocytes of adult *C. retropictus* in the presence of adult's plasma but not in the absence of the plasma [11]. Therefore, hemocytes of adult, and also presumably juvenile, *C. retropictus* would phagocytose the organism if they could adsorb it. Hemocytes of juvenile *N. albicilla*, in comparison, were found to be attracted chemotactically to *V. parahaemolyticus* regardless of the presence of *N. albicilla* plasma [6] and active in lysosomal enzymes [9], suggesting that *N. albicilla* would have developed full activities of hemocytes in the juvenile stage [3]. Conclusively, delayed maturation of *C. retropictus* hemocytes in functions related with inner structures of the hemocytes would be responsible for the low recognition of juvenile's hemocytes to *V. parahaemolyticus*.

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