Migratory Responses of Hemocytes to *Vibrio parahaemolyticus* in the Alimentary Tract of an Estuarine Neritid Gastropod, *Clithon retropictus*

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ABSTRACT. Migratory responses of hemocytes to *Vibrio parahaemolyticus* strain D3 in the alimentary tracts of an estuarine neritid gastropod, *Clithon retropictus*, and a related marine neritid, *Nerita albicilla*, were examined under the scanning electron microscope. After ingesting the strain, active responses were seen at the esophagus, stomach and anterior intestine of adult *C. retropictus* and at the middle and posterior intestines of adult *N. albicilla*. When the alimentary tracts were isolated from the gastropod and incubated in *vitro* with strain D3, active response was induced at the most parts of the tract of the adult gastropods and at the stomach and the anterior intestine of juvenile *C. retropictus*. The responding hemocytes were confirmed to be granulocytes in the semi-thin sections of the tract of adult *C. retropictus*. The poor hemocyte responses at the middle and posterior intestines of juvenile *C. retropictus* might support the colonization of the organism there.

KEY WORDS: alimentary tract, *Clithon retropictus*, hemocyte, migration, *Vibrio parahaemolyticus.*


An estuarine neritid gastropod, *Clithon retropictus*, is an important reservoir of an enteropathogenic organism, thermostable direct hemolysin (TDH)-producing strain of *Vibrio parahaemolyticus* [3, 4]. TDH-producing strain D3 was found to survive at a level of $10^4$–$10^5$ and $10^6$ viable units/g for up to 21 days in the alimentary tract of juvenile and adult *C. retropictus*, respectively, ingested the strain and maintained in ultraviolet (UV)-irradiated recirculating artificial seawater with a salinity of 20‰ at 28°C [5]. However, the strain was eliminated from 2 marine neritid gastropods, *Nerita albicilla* and *Heminerita japonica*, maintained in 35‰ artificial seawater within 4 days [8,10]. Hemocytes of adult *C. retropictus* and adult and juvenile *N. albicilla* were attracted to strain D3 chemotactically in modified Chernin’s balanced salt solution (CBSS), which was enhanced significantly in the presence of the respective molluscan plasma [6, 9]. In contrast, hemocytes of juvenile *C. retropictus* were attracted to strain D3 chemotactically in the presence of *C. retropictus* plasma but not in the absence of the plasma [6]. From these findings, *V. parahaemolyticus* was expected to induce active migratory responses of the hemocytes at the epithelia of the alimentary tract in adult and juvenile *N. albicilla* and adult *C. retropictus*, but not in juvenile *C. retropictus*. The present study characterizes the hemocyte response to *V. parahaemolyticus* in the alimentary tract of juvenile *C. retropictus*.

MATERIALS AND METHODS

Adult and juvenile *C. retropictus*, 12–20 and 5–8 mm in shell height, respectively, were collected at estuaries in Okinawa Island. Adult *N. albicilla*, 10–15 mm in shell height, was collected at the coral reef of Okinawa Island. *C. retropictus* and *N. albicilla* were maintained in UV-irradiated recirculating conditions of fresh water and 35‰ artificial seawater [3, 7], respectively, at room temperature for 7 days and confirmed non-detectable for *V. parahaemolyticus*.

*C. retropictus* and *N. albicilla* were then incubated in the suspension of *V. parahaemolyticus* strain D3 at a level of $10^6$ viable units/ml in 20 and 35‰ artificial seawater, respectively, at 25°C for 24 hr with aeration to allow ingestion of the strain. The gastropods were then washed with distilled water to remove the strain attached to the surface and maintained in UV-irradiated recirculating 20 and 35‰ artificial seawater, respectively, at 25°C for 2 days [3, 10]. Cultured diatom algae were fed to the gastropods throughout the study [3]. The gastropods were sacrificed at appropriate times. Alimentary tracts were isolated from the gastropods, rinsed and fixed in 2.5% glutaraldehyde buffered with 0.15 M veronal (pH 8.6) at 4°C for 4 hr, rinsed in the same buffer and postfixed in 1% osmium tetroxide in the same buffer. The samples were dehydrated in a graded series of ethanol followed by t-butyl alcohol, freeze-dried and coated with gold-platinum and observed under a scanning electron microscope S-2450N (Hitachi) (SEM) [11].

Alimentary tracts were then fixed and processed for SEM observation as described above. The alimentary tracts were isolated from adult *C. retropictus* that had ingested strain D3 as described above, dissected and fixed in 2.5% glutaraldehyde in 0.15 M veronal buffer (pH 8.6) for 2 hr at 4°C, rinsed in the same buffer, postfixed in 1% osmium tetroxide for 2 hr, dehydrated through an ethanol series and embedded in Epon 812 resin. Semi-thin sections were stained with 0.5% toluidine blue in 0.1% sodium carbonate and examined with an optical
RESULTS

Migratory responses of the hemocytes at the alimentary tracts of adult *C. retropictus* and adult *N. albicilla* after the ingestion of strain D3 are summarized in Table 1. Many hemocytes were seen on the epithelia of the esophagus, stomach (Fig. 1) and the anterior intestine of *C. retropictus* and of the middle and posterior intestines of *N. albicilla* (Fig. 2) just after the ingestion. The hemocyte response was seen at the anterior intestine of *C. retropictus* 2 days later (Fig. 3) but not at any part of the tract of *N. albicilla* 12 hr later. Most hemocytes seen on the epithelia of these gastropods were withered.

Alimentary tracts were isolated from *V. parahaemolyticus*-free gastropods, incised lengthwise, incubated in vitro with strain D3 and observed under SEM (Table 2). Many hemocytes were seen at the most parts of the tract, especially at the stomach (Fig. 4) and intestine in adult *C. retropictus*. On the other hand, the hemocyte response was seen only at the stomach and anterior intestine in juvenile *C. retropictus* (Fig. 5). Micro-colonies of rod-shaped bacteria were seen on the epithelium of the intestine in juvenile *C. retropictus* (Fig. 6). The response was seen throughout the tract in adult *N. albicilla*. Few hemocytes were seen on the epithelia of the most parts of the tracts in the uninoculated gastropods.

Alimentary tract of adult *C. retropictus* that had ingested strain D3 was sectioned, stained with toluidine blue and observed under the optical microscope. Hemocytes with large cytoplasmic granules were seen in the epithelium of the middle intestine and in the connective tissue under the epithelium (Fig. 7). The hemocyte response was not seen in the uninoculated samples.

DISCUSSION

Active migratory responses of the hemocytes were induced in the alimentary tracts of adult *C. retropictus* and adult *N. albicilla* after the ingestion of *V. parahaemolyticus* strain D3. The response lasted at least 2 days in *C. retropictus* and less than 12 hr in *N. albicilla* (Table 1). The response was weak just after the ingestion and invisible 2 days later at the posterior intestine, possible colonization site of the strain, in *C. retropictus*. Strain D3 was eliminated rapidly from the alimentary tract of adult *N. albicilla* [10] though it survived at a low level in adult *C. retropictus* [5]. The weak responses at the posterior intestine seem to be concerned in the survival of the strain in these gastropods.

### Table 1. Migration of hemocytes onto the epithelium of the alimentary tracts of two adult neritid gastropods, *C. retropictus* and *N. albicilla*, after ingestion of *V. parahaemolyticus* D3

<table>
<thead>
<tr>
<th></th>
<th>Mouth</th>
<th>Esophagus</th>
<th>Stomach</th>
<th>Anterior</th>
<th>Intestine</th>
<th>Posterior</th>
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<tr>
<td><em>C. retropictus</em></td>
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<td>just after ingestion</td>
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<td><em>N. albicilla</em></td>
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<td>just after ingestion</td>
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<td>12 hr after ingestion</td>
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</table>

Number of hemocytes per 0.01 mm² of the epithelium; –: 0, ±: 1–10, +: 11–50, ++: 51–100, +++: more than 101.

### Table 2. Migration of hemocytes onto the epithelium of the alimentary tract of two neritid gastropods, *C. retropictus* and *N. albicilla*, after incubation of the removed alimentary tracts with *V. parahaemolyticus* D3 at 25°C for 2 hr in modified Chernin’s balanced salt solution

<table>
<thead>
<tr>
<th></th>
<th>Mouth</th>
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<th>Stomach</th>
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<tr>
<td><em>C. retropictus</em></td>
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<td>Juvenile</td>
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<td><em>N. albicilla</em></td>
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<tr>
<td>Adult</td>
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</table>

Number of hemocytes per 0.01 mm² of the epithelium; –: 0, ±: 1–10, +: 11–50, ++: 51–100, +++: more than 101.
Hemocytes of the adult gastropods showed chemotaxis to strain D3 in CBSS, which was enhanced by plasma of the respective gastropods [6, 9]. If the hemocytes would phagocytize the strain in the hemocoeil and migrate afterwards to the alimentary cavity, the response would be seen throughout the tract simultaneously. Indeed, it was seen all over the tract simultaneously in the adult gastropods when the removed tracts were incubated with the strain in vitro (Table 2). In comparison, main sites of the responses in C. retropictus were seen at esophagus, stomach and anterior intestine just after the ingestion but were not seen at the esophagus and stomach 2 days after the ingestion in the in vivo study (Table 1). Thus the responses seem to be ready all over the tract and to be induced by the ingested strain in the adult gastropods.
Hemocytes of juvenile *C. retropictus* were reported to migrate to strain D3 chemotactically only in the presence of *C. retropictus* plasma [6] so that the hemocyte response was expected to be poor on the removed alimentary tract. However, it was active at the stomach and anterior intestine and poor at the middle and posterior intestines in the removed tract (Table 2). Although strain D3 survived in the alimentary tract of the juvenile, the site of colonization was not confirmed in our previous study [5]. Poor hemocyte response at the lower intestine seems to support the colonization of the strain there. It is, however, uncertain the reason why the response was seen only at the stomach and the anterior intestine in juvenile *C. retropictus* in the present study.

Many rod-shaped bacteria were seen on the epithelia of the test specimens (Fig. 6). Some indigenous organisms were seen on the epithelia of the tract incubated *in vitro* with strain D3 (Figs. 4 and 5). C. *retropictus* and *N. albicilla* were maintained in 20 and 35‰ artificial seawater, respectively, in the present study. Mean salinity of the hemolymph was reported to be 12 and 32‰ for *C. retropictus* and *N. albicilla*, respectively [6, 9]. In fact, forms of the hemocytes seen on the removed tracts (Figs. 4 and 5) were similar to those cultivated *in vitro* [11]. Thus the salinities of water maintaining the gastropods might be rather high for the hemocytes to keep the typical forms.

Many granulocytes were seen in the epithelia of the intestine of adult *C. retropictus* after ingestion of strain D3 (Fig. 7). Most of the molluscan hemocytes are known to be granulocytes with cytoplasmic granules [13]. Thus the cells seen on the epithelia under SEM were concluded to be granulocytes.

There are few morphological studies on the colonization of *Vibrio* and the hemocyte responses to the organism at the alimentary tract of aquatic invertebrates. Huq *et al.* [1] reported the colonization of *Vibrio cholerae* in the hindgut of the blue crab, *Callinectes sapidus*. *V. cholerae* and *V. parahaemolyticus* were shown to adhere to chitin [2, 12]. Ectodermic hindgut of the crustacean is covered with chitin so that *V. cholerae* seems to attach to the chitinous surface of the hindgut of the crab [1]. Mechanism of *V. parahaemolyticus* strains to colonize on the endodermic mucous membrane of *C. retropictus* intestine would be analyzed in near future.

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REFERENCES


