In Vitro Attachment of Vibrio parahaemolyticus to Hemocytes of Two Gastropod Molluscs

Noriclack H. KUMAZAWA, Takahiko TANIGAWA1, Yoshinori TANAKA1, Hitoshi OSATAKE2, and Keiichi TANAKA2

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

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ABSTRACT. Vibrio parahaemolyticus D-3 was observed to attach to hemocytes of a marine gastropod mollusc, Nerita albicilla, regardless of the presence of N. albicilla serum. The organism attached to hemocytes of an estuarine gastropod, Clithon retropticus, in the presence of C. retropticus serum while the attachment to the hemocytes was decreased significantly in the absence of the serum. These evidences suggest that N. albicilla hemocytes would facilitate the clearance of V. parahaemolyticus from the alimentary tract of the mollusc and that C. retropticus hemocytes would protect C. retropticus against the invasion of V. parahaemolyticus to hemocoele of the mollusc.—KEY WORDS: attachment, hemocyte, mollusc, Vibrio parahaemolyticus.


Vibrio parahaemolyticus is an estuarine organism which can cause gastroenteritis in man. The organism was found to survive in an estuarine gastropod mollusc, Clithon retropticus, but not in a marine gastropod, Nerita albicilla, in our previous study [5]. In vitro study showed that C. retropticus hemocytes were weak in migratory responses to the organism compared with N. albicilla hemocytes [6]. In addition, a role of C. retropticus serum to the migratory activity of hemocytes was suggested in our preliminary study [9]. As migration and attachment of the hemocytes to the organism are thought to be essential processes for the hemocytes to phagocitize the organism, an attachment of V. parahaemolyticus to the hemocytes and its stimulation by the mollusc can serum were investigated as described in the present study.

MATERIALS AND METHODS

N. albicilla and C. retropticus were maintained in artificial seawater with salinities of 35 and 15 permil, respectively [5]. Throughout the study, phosphate buffered saline (PBS) with 3% NaCl (pH 7.8) and PBS with 1.5% NaCl (pH 7.4) were used for experiments of N. albicilla and C. retropticus, respectively. The mollusc hemocytes were collected and suspended in PBS-3% NaCl or PBS-1.5% NaCl at a concentration of 10⁶ cells per ml as described previously [6].

V. parahaemolyticus D-3, thermostable direct hemolysin-producing strain, grown in Heart Infusion Broth (Nissui) supplemented with 3% NaCl at 37°C for 18 hr, was harvested by centrifugation at 3,000 rpm for 20 min. The sediment was washed with PBS-3% NaCl or PBS-1.5% NaCl and resuspended in each solution at a density of 10⁶ viable units per ml.

N. albicilla and C. retropticus hemocytes were incubated with the organism at a concentration of 5 × 10⁵ cells per ml and 5 × 10⁷ viable units per ml, respectively, in PBS-3% NaCl or PBS-1.5% NaCl or in those with 10% sera of each molluscs at 25°C for 3 hr.

After the incubation, the hemocytes were rinsed with each solution, fixed with methanol, stained with Gram’s method and calculated the organism attached to 100 hemocytes under a light microscope followed by the manner reported previously [7]. The experiments were repeated three times.

The incubated hemocytes were also rinsed and fixed with 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.3), and postfixed with 1% osmium tetroxide in the same buffer. After conductive-staining with 2% tannic acid and 1% osmium tetroxide, they were dehydrated in a graded series of ethanol, immersed to t-butyl alcohol and freeze-dried by the t-butyl alcohol freeze-drying method [4]. The dried samples were coated with platinum using an ion-sputtering coater and observed under a field emission scanning electron microscope HFS-2ST (Hitachi).

RESULTS

The number of V. parahaemolyticus attached to
hemocytes of C. retropictus and N. albicilla with and without the respective molluscan sera were shown in Table 1. The organism attached to N. albicilla hemocytes regardless of the presence of the molluscan serum. The number of organisms attached to C. retropictus hemocytes in the presence of C. retropictus serum was significantly higher than the number of organisms attached to the hemocytes in the absence of the serum (p<0.05).

Scanning electron micrographs of the hemocytes showed that C. retropictus hemocytes were attached by few organisms in the absence of the molluscan serum (Fig. 1a) but many in the presence of the serum (Fig. 1b). Many organisms were seen on N. albicilla hemocytes regardless of the presence of the molluscan serum (Figs. 2a and 2b).

**DISCUSSION**

V. parahaemolyticus D-3 was found to attach to N. albicilla hemocytes regardless of the presence of the molluscan serum (Table 1, Fig. 2). As N. albicilla hemocytes were known to migrate to V. parahaemolyticus suspensions in artificial seawater [6], the hemocytes were confirmed to migrate and attach to the organism even in the absence of the serum. V. parahaemolyticus has been already shown to be cleared from N. albicilla within 3 days [5]. These evidences suggest that the hemocytes might play a role in clearing the organism from the alimentary tract of N. albicilla.

Recognition and attachment of foreign particles by some molluscan hemocytes were shown to be facilitated by serum factors [1, 10, 11, 12]. C.

<table>
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<tr>
<th>Serum concentration (%)</th>
<th>Number of V. parahaemolyticus cells attached to 100 hemocytes$^a$</th>
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<tbody>
<tr>
<td></td>
<td>C. retropictus hemocytes</td>
</tr>
<tr>
<td>0</td>
<td>163±13$^b$</td>
</tr>
<tr>
<td>10</td>
<td>600±78</td>
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</table>

$^a$ C. retropictus and N. albicilla hemocytes were suspended in PBS with 1.5 and 3.5% NaCl.

$^b$ V. parahaemolyticus cells were incubated with the hemocytes at a concentration of 5$x10^3$/ml and 5$x10^7$/ml respectively at 25°C for 3 hr (mean±SD).

Fig. 1. Attachment of V. parahaemolyticus D-3 to C. retropictus hemocytes in the absence (a) and the presence (b) of C. retropictus serum.
retropictus hemocytes spreading pseudopodia with many spikes [8] were attached by V. parahaemolyticus D-3 cells in the presence of the molluscan serum (Table 1, Fig. 1). These evidences suggest that the hemocytes would protect the invasion of V. parahaemolyticus to hemocoel in C. retropictus.

Few evidences have been elucidated on the strategies of some pathogens against the host molluscs. Fischer [3] showed that hemocytes of the flat oyster, Ostrea edulis, and the cupped oyster, Crassostrea gigas, did not differ significantly in their ability to bind latex beads and that C. gigas hemocytes were able to bind more Bonamia ostreae, a protozoan parasite of O. edulis, than O. edulis hemocytes. Though B. ostreae might have a mechanism of evading the protective system of O. edulis, the effects of the oyster sera to these hemocytes are not clear because the experiments were done only in the presence of the respective molluscan sera. Our preliminary study [7] showed that latex beads attached to hemocytes of N. albicilla and C. retropictus in similar levels. Therefore, hemocytes of C. retropictus and N. albicilla seem to be functional against the latex beads in a similar manner. The reason why C. retropictus hemocytes are less active to V. parahaemolyticus is not known. Factors contributing to recognition and destruction of the organisms in the molluscan immune system should be elucidated to understand the poor attachment of V. parahaemolyticus to C. retropictus hemocytes.

The incubation was done in PBS instead of balanced salt solution [2], in which the attachment was observed in low level of the hemocyte metabolism. However, it was not confirmed whether the organism would be phagocytized into the hemocytes in this study. Phagocytosis of the organism by the hemocytes will be analyzed in the balanced salt solution.

REFERENCES


