Response of innate immune factors in abalone Haliotis diversicolor supertexta to pathogenic or nonpathogenic infection.

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ABSTRACT Cell free hemolymph from Haliotis diversicolor supertexta was prepared from fluid collected at 1, 4, 8, 12, 24, 48, 96 h after injection with either Escherichia coli, Vibrio parahaemolyticus or 0.9 NaCl solution (control group). The response of selected innate immune parameters (lysozyme, antibacterial activity, alkaline phosphatase, acid phosphatase, phenoloxidase, and superoxide dismutase) was investigated. Results showed that the activities of ACP (Acid Phosphatase) from abalone injected with V. parahaemolyticus were much higher than that of the control group at 24 h after injection. The ALP (Alkaline Phosphatase) activities of abalone challenged with V. parahaemolyticus were significantly higher than those of the control group at 8 h and increased further up to 48 h after the challenge. In contrast, the activities of ALP and ACP in the E. coli-challenged group showed no statistically significant differences at any of the sampling times. The activities of SOD (Superoxide Dismutase) in cell free hemolymph from the V. parahaemolyticus-exposed group were significantly lower than those of the control group at both 1 h and 24 h, whereas there was no difference in SOD activity observed in the group exposed to E. coli at any of the sampling times. The activities of lysozyme and phenoloxidase in Haliotis diversicolor were relatively low in both control and bacteria-exposed groups when compared with reports for other invertebrates: no significant difference was found between the infected groups and the control for these two parameters, due to the low activities and high individual variance.

KEY WORDS: Haliotis diversicolor supertexta, superoxide dismutase, innate immune, acid phosphatase, alkaline phosphatase

INTRODUCTION

Having an open circulation system, abalone has considerable opportunity to encounter the pathogens and pollutants present in its surrounding water. Although little is known about the innate defense system(s) of abalone, some information is available from the study of other molluscs. To cope with the foreign invaders, many bivalves possess cellular and extra cellular defense mechanisms that are remarkably effective. Hemocytes are the main cellular effectors of invertebrate immunity and are capable of recognizing pathogens in a lectin-mediated process (Pipe 1990, Renwratz 1983) and in phagocytosing, thus isolating and inactivating them (for a review see: Canesi et al. 2002). In addition, cell-free hemolymph of

certain bivalves has been shown to contain various biologically active substances involved in innate defense, including agglutinins (e.g., lectins), lysosomal enzymes (e.g., acid phosphatase, lysozyme), toxic oxygen intermediates, and various antimicrobial peptides (Canesi et al. 2002).

In abalone, some research has been focused on the cellular defense system. Lebel et al. (1996) have established that two types of hemocytes are present in the blood of the abalone Haliotis turbeculata. Moreover, it has also been shown that salinity stress and certain pollutants can affect the migratory and phagocytic activities of these hemocytes (Chen 1996, Martello et al. 2000). Recently, Shelagh et al. (2003) have shown a direct link between stress and the immune reaction in the abalone H. tuberculata using immune parameters such as the number of circulating hemocytes, the migratory activity, the phagocytic capacity, and the respiratory burst responses of the hemocytes.

H. diversicolor supertexta is one of the most commercially important cultured abalone in southern China. However, although progress in abalone culture has occurred in recent years, abalone culture remains an expensive undertaking, due in part to the catastrophic effects of frequent infectious disease outbreaks (Zhang et al. 2001). To understand the mechanism of innate immunity by which H. diversicolor supertexta defends itself against pathogens, we infected H. diversicolor supertexta with a nonpathogenic bacterium (Escherichia. coli) as well as a pathogenic bacterium (Vibrio. parahaemolyticus) and compared several immunity-related parameters at different exposure times.

MATERIALS AND METHODS

Animals, Immune Challenge, and Hemolymph Collection

Abalone (Haliotis diversicolor supertexta) were collected from a commercial farm (Futian, Dongshan, Fujian Province) and maintained in polyethylene tanks, each containing 20 animals in 50 L of aerated and sand-filtered seawater at 23[degrees]C to 25[degrees]C. Animals were left undisturbed for 2 wk to acclimate to their environment. Abalone were challenged by injecting either 50 L of Vibrio parahaemolyticus (isolated from diseased abalone, Zhang et al. 2001) in 0.9% NaCl (6.7 x l07 cells/mL), 50 L of E. coli in 0.9% NaCl (6.7 x 107 cells/mL) or 50 L of 0.9% NaCl (as control) into their pleopod muscle. After injection, the abalone were returned to their original tanks containing seawater at the same temperature. Hemolymph (approximately 1.0 mL/animal) from unchallenged or challenged abalone was collected at 1, 4, 8, 12, 24, 48, 96 or 192 h post challenge using a capillary to withdraw fluid from a center incision in the pleopod muscle. Samples were collected into a 1.5 mL centrifugal tube and immediately centrifuged at 150g for 5 min at 4[degrees]C to remove hemocytes. Cell-free hemolymph (CFH) samples were frozen at ~80[degrees]C until used.

Biochemical Assays

ACP and ALP activity assays were carried out according to King's method (Cui 1981) using a commercial kit (Nanjing Jiancheng Bioengineer research institute, China). Analysis was performed in 96-well flat-bottomed microtitre plates and the concentrations of products were monitored spectrophotometrically at 550 nm after incubation of the plate at 37[degrees]C for 30 min in the case of ACP, or for 20 min at 37[degrees]C for ALP. The activity of ACP

and ALP was defined as the amount of phenol (mol) produced under the mentioned conditions per mg protein.

SOD activity assay, a modification of the xanthine-xanthine oxidase assay (McCord & Fridovich 1969) was adapted by using a kit (Nanjing Jiancheng Bioengineer Research Institute, China). The assay was carried out in 96-well flat-bottomed microtitre plates and 1 nitrite unit (NU) of SOD was defined as the quantity of enzyme (per mg protein) that inhibits the reduction of tyrochrome c by 50%.

Phenoloxidase activity was measured according to the methods of Horowitz (1952) and Ashida (1971) with L-Dopa used as the substrate. Briefly, 20 [micro]L of cell-free hemolymph samples were added to the wells of a 96-well flat-bottomed microtitre plate in triplicate. To each well, 200 [micro]L of PBS (0.1 mol/L, pH = 6) and 20 [micro]L of L-Dopa solution (0.01 mol/L), were added and the absorbance was read continuously at 490 nm for 15 min. One unit (PO activity) was defined as absorbance change in 490 nm of 0.001 in 1 min and the concentration was expressed as units of activity per mg of protein contained in the sample.

Lysozyme activity was analyzed according to Wilson and Ratcliffe (2000). Briefly, purified lysozyme (100,000 units/mg; Sigma) was diluted in PBS pH 6.2 to give the standards of 0, 2, 4, 8, 12, 16, 20, and 30 g/mL. Aliquots (10 L) of each standard, or the CFH samples were added to the wells of a 96-well flat-bottomed microtitre plate in triplicate. To each well, 250 L of a suspension of lyophilized Micrococcus luteus in PBS (0.3 mg/mL; Sigma) was added and the absorbance was continuously monitored at 450 nm for 10 min. For the calculation of lysozyme-like activity, the values for the standards were plotted on a graph of D[A.sub.450 nm]/10 min against lysozyme activity (1 unit-change in [A.sub.450 nm] of 1.0 in 1 min). The values for lysozyme-like activity for each cell-free hemolymph sample were extrapolated from this graph and the concentration was expressed as units of activity per mg protein contained in the sample.

Total protein content of each cell-free hemolymph sample was determined according to Bradford (1976) using bovine serum albumin (BSA) as a standard.

Statistical Analyses

All data are presented as means and standard errors of at least 20 specimens. For comparison of two means, paired or unpaired Student t-tests were used where appropriate. $P < 0.05$ was considered as the lower limit of significance, whereas $P < 0.01$ was considered as the most significant difference.

RESULTS

Time Course of ALP and ACP Activities in Cell Free Hemolymph Following Bacterial Challenge

The activities of ACP from abalone injected with V. parahaemolyticus were significantly higher than that of control group at 24 h after injection (Fig. l). In contrast, the activities of ACP between E. coli group and control group demonstrated no signifi cant difference at each sampling time (Fig. 1). All abalone died at 192 h after injection and no data were

available at that time.

[FIGURE 1 OMITTED]

The ALP activities of abalone injected with V. parahaemolyticus were significantly higher than that of the control group at 8 h ($P < 0.05$) and were even more significant at 48 h ($P < 0.01$). Interestingly, at 96 h post exposure the ALP activities of the V. parahaemolyticus-challenged group were notably lower than that of control and E. coli group (Fig. 2). Same as ACP, the activities of ALP between E. coli group and control group demonstrated no significant difference at each sampling time (Fig. 2).

[FIGURE 2 OMITTED]

Time Course of SOD Activities in Cell Free Hemolymph Following Bacterial Challenge

The activities of SOD in cell free hemolymph from V. parahaemolvticus group were significantly lower than that of control group at 1 h and 24 h. However, there was no statistical difference between the E. coli-challenged group and control group at any sampling time when the activities of SOD were considered (Fig. 3). Moreover, as a general trend, the average of the SOD activity from the V. parahaemolyticus group was lower than that of control and E. coli group at each sampling time.

[FIGURE 3 OMITTED]

Time Course of Lysozyme Activities in Cell Free Hemolymph Following Bacterial Challenge

No significant differences in the activities of lysozyme were observed under any of the exposure conditions and at any of the times of exposure. The degree of variation, in the cell-free hemolymph samples between individual abalones was, however, quite large (e.g., 24 h after injection with 0.9% NaCl, the samples ranged from 0.015-0.472 U/mg) and any underlying trend may have been masked by this extreme variability (Fig. 4).

[FIGURE 4 OMITTED]

Time Course of Phenoloxidase Activities in Cell Free Hemolymph Following Bacterial Challenge

The activities of phenoloxidase in Haliotis diversicolor supertexta were low (Fig. 5), considerably lower than that reported in other invertebrates. For example, values of 2.41-3.46 have been reported in scallop Chalyms ferreri (Sun & Li 1999). No significant differences were observed under any of the exposure conditions and at any of the times of exposure.

[FIGURE 5 OMITTED]

DISCUSSION

A series of immune defense reactions will normally be elicited if bacteria or other pathogenic microbes enter the body of an invertebrate (Cheng 1978). Because

invertebrates do not have the capacity to mount humoral and adaptive immune responses, hemocytes play an important part in the shellfish defense system because of their ability to phagocyte, encapsulate, and kill microbes (Zhou & Pan 1997). In addition, processes such as agglutinins, lysosomal enzymes, and toxic reactive oxygen intermediates form during a respiratory burst and may also play an essential role in innate immune responses to clean invading pathogens from the shellfish tissue and hemolymph (Aswanik et al. 1999, Mitta & Vandenbulcke 1999, 2000).

ACP is an important component of the phagocytic lysosome, and is released during the process of phagocysis and encapsulation (Zhai et al. 1998). Mollusc ACP is found mainly in granulocytes and is responsible for lysis and eventual decomposition of foreign agents including many bacteria. It has previously been shown that the concentrations of lysosome enzymes in some shellfish were increased after they were infected by bacteria (Cheng 1979). In the present study, the activities of ACP and ALP in V. parahaemolyticus group were significantly increased at the time points before 24 h and 48 h of the exposure respectively.

These results indicated that V. parahaemolyticus induced the ACP and ALP activities and activated the immune defense response in abalone, Haliotis diversicolor supertexta. However, the E. coli had no effects on ACP and ALP activity in our study, because we observed no significant differences to results obtained from the control group. Our results are in contrast to those reported by Sun and Li (1999) who observed that E. coli can also significantly induce ACP and ALP activities in the scallop, Chalyms ferreri.

It has been previously suggested by Feng (1988) that the elevation of lysosome enzyme (including lysozyme, ACP and ALP) activity was not only one mechanism of immune defense, but that its depletion may also be a symptom of disease. In the present study, ALP activities declined noticeably at 96 h and ACP activity was substantially decreased at 48 h post injection when the abalone were exposed to V. parahaemolyticus. Thus, it may be suggested that the capability of the immune defense in abalone can be disturbed by this pathogenic bacterium and may further be an indicator of the severity of infection in this animal. It was noteworthy that we found some evidence of pathologic changes in abalone injected with V. parahaemolyticus (data not shown). Symptoms of the diseased abalone included increased mucus, decreased activity, and loss of absorbability. Furthermore, no samples were available at 192 h after injection due to the high mortality in the V. parahaemolyticus injected group (no mortality was observed in either the control or E. coli exposed group).

Superoxide dismutase is capable of eliminating superoxide free radicals and may prove protective in bivalves, freeing them from the impact of reactive oxygen species (Nasci et al. 2002). In general, SOD will be induced when the amount of free radical increased. The activities of SOD in cell free hemolymph from the V. parahaemolyticus-challenged group were significantly lower than that of the control group at 1 h and 24 h in the present study. Our results are in agreement with the previously reported results of Ding et al. (1996), who infected Haliolis discus hanna with E. coli or Vibrio spp. and found that the SOD activity in cell-free hemolymph was markedly depleted. These phenomena are so called negative-induced, which may also be an indication of immune defense activation in abalone. The reducing of SOD activities allows the relative long existence of reactive oxygen intermediates and is helpful against bacteria. Again, as was observed for the ALP and ACP

responses, there was no significant difference between the E. coli-treated group and the control when SOD in cell-free hemolymph was considered. These results may indicate that abalone immune defense shows different responses to pathogenic and nopathogenic bacteria, although the mechanism by which they might be capable of distinguishing such differences remains unclear (V. parahaemolyticus is a conditional pathogenic bacterium, it caused abalone disease under our experiment condition. E. coli, in general, is a natural bacterium and had no effects on abalone in our study).

The activities of lysozyme, and phenoloxidase in Haliotis diversicolor supertexta were low. For example, lysozyme activity of 4 [+ or -] 0.6 units/mg protein was recorded in cell-free hemolymph of Blaberus discoidalis (Wilson & Ratcliffe 2000). While in abalone, Haliotis diversicolor supertexta, the mean value was 0.2 units/mg protein below. As mentioned in Results, the activities of phenoloxidase in Haliotis diversicolor supertexta were 10 times lower than in the scallop Chalyms ferreri (Sun & Li 1999). Therefore, it may be that these two parameters may play a less important role in immune defense in abalone. High variability was observed between individual samples.

Further work is required to ascertain the role of these enzymes in abalone immune defense. Fortunately our research group has cloned the SOD gene in abalone, Haliotis diversicolor supertexta, and further research to determine why and how the SOD activity is diminished, following challenge by such pathogenic bacteria, is ongoing. Genomic scale investigation is also being carried out in our laboratory on the molecular mechanism of abalone response to pathogenic and no-pathogenic bacteria by using a combination of cDNA microarray and other technology.

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