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ENHANCEMENT OF MICROBICIDAL ACTIVITY IN THE TIGER SHRIMP *PENAEUS MONODON* VIA IMMUNOSTIMULATION

H. H. Sung, Y. L. Yang, and Y. L. Song

ABSTRACT

We studied the clearance ability of hemolymph drawn from the tiger shrimp *Penaeus monodon* immersed in a viable cell suspension of *Vibrio vulnificus*. Results show that *Vibrio* cells were largely eliminated from shrimp hemolymph within 12 h following invasion and completely undetectable at 24 h. We also examined the anti-*E. coli* activity of plasma, phenoloxidase (PO) activity, as well as the production of superoxide anion (O_2^-) by shrimp hemocytes following treatment with each of these 3 immunostimulants. Resultant survival indexes show that some plasma, but not all, exhibits anti-*E. coli* activity. However, such an activity in all plasma tested could be enhanced by beta-glucan or zymosan up to 24 h following treatment, and slightly enhanced at 6 h following treatment with a *Vibrio* antigen. PO activity and O_2^- generation were stimulated by all 3 immunostimulants. Enhanced PO activities were observed from 5 min to 24 h following treatment, with the highest activity occurring at 3 h; the activity recovered to normal on day 3. Increased O_2^- production was observed from 3–12 h in both glucan- and zymosan-treated shrimp, and at 6 h in shrimp treated with *Vibrio* antigen. These results show that: (1) some microbicidal reactions may be involved in the clearance of invasive *V. vulnificus* from shrimp hemolymph; (2) the 3 immunostimulants have the ability to enter shrimp via immersion treatment, thus leading to activation of both the plasma-related factors and hemocytes and the strengthening of PO activity and O_2^- production; and (3) these enhanced microbicidal reactions exhibit a rapid response that is short in duration.

Decapod crustaceans have the capability of rapidly clearing invading bacteria from their hemolymph (Cornick and Stewart, 1968; McKay *et al.*, 1969; McKay and Jenkin, 1970; Smith and Ratcliffe, 1980; White and Ratcliffe, 1982; McCumber and Clem, 1983; Adams, 1991; Martin *et al.*, 1993). Examples have been reported of lobsters, shore crabs, and penaeid shrimps removing in excess of 75% of bacterial cells within 10 min to 1 h after injection (Cornick and Stewart, 1968; White and Ratcliffe, 1982; Adams, 1991; Martin *et al.*, 1993). Both humoral and cellular responses are thought to be involved in this clearance reaction. Crustaceans possess a wide variety of noncellular factors that are both naturally occurring, inducible, bioactive molecules (e.g., agglutinin, killing factors, lysins, precipitins, and clotting agents) (Smith and Chisholm, 1992). Some antibacterial factors have been found in lobster plasma, such as bactericidins, which were perceived as being effective against a variety of bacteria in lobsters (Cornick and Stewart, 1968; Stewart and Zwicker, 1972; Mori and Stewart, 1978).

The circulating hemocytes plus tissue phagocytes, which are primarily found in crustacean gills, have been implicated in the clearance of invading microorganisms (Smith and Ratcliffe, 1980). These cells remove foreign particles in the crustacean hemocoel by phagocytosis (McKay and Jenkin, 1970; Fontaine and Lightner, 1974; Paterson and Stewart, 1974; Paterson *et al.*, 1976; Smith and Ratcliffe, 1978, 1980; Goldenberg *et al.*, 1984), or hemocyte encapsulation which is initiated by the prophenoloxidase activating system (Unestam and Söderhäll, 1977; Söderhäll, 1982; Smith and Söderhäll, 1983; Hose *et al.*, 1990).

Many crustacean researchers have documented that the prophenoloxidase (proPO) activating system functions in nonself recognition and host defense (Söderhäll, 1982; Ratcliffe *et al.*, 1985; Smith and Söderhäll, 1991). More importantly, proteins that are associated with the proPO system have been shown to be directly involved in the communication between hemocytes and also in the elimination of foreign particles, such as parasites, within the body cavity of crayfish (Söderhäll *et al.*, 1994). In addition, some microbial products, such as beta-

1,3-glucan from fungal cell walls, and lipopolysaccharide and peptidoglycan from bacterial cell walls have been recognized as elicitors of the proPO system (Unestam and Söderhäll, 1977; Söderhäll and Unestam, 1979; Yoshida and Ashida, 1986; Söderhäll *et al.,* 1990). Our previous study also showed that the proPO activating system of shrimp hemocytes could be enhanced by in vitro beta-glucan treatment (Sung *et al.,* 1994). In addition, Song and Hsieh (1994) demonstrated in vitro a respiratory burst in shrimp hemocytes which was thought to be related to phagocytosis.

The aim of the present research was to investigate the relative microbicidal reactions associated with the clearance of bacteria, as well as to evaluate the stimulative effects of three immunostimulants applied to shrimp in vivo.

Materials and Methods

Experimental Animals.—Tiger shrimp (*Penaeus monodon* Fabricius), each weighing 30–40 g, were purchased from a local market and acclimated to 2.5% salinity/25°C pond water in a 120-l plastic container for 2 days prior to our experiments. The stocking density was 4 individuals per l.

Preparation of Bacterial Suspension.—The TG617 strain of *Vibrio vulnificus* (see Song *et al.,* 1990) used for the clearance test was cultured overnight in tryptic soy broth (TSB containing 3% NaCl) at 28°C. Before immersion treatment, the concentration of bacterial suspension was adjusted to 10^7 CFU (colony forming unit) per ml of pond water. In addition, the V517 strain of *Escherichia coli* (see Macaina *et al.,* 1978) was cultured overnight in TSB (tryptic soy broth, Difco) at 37°C, after which it was subcultured in 10 ml of TSB. After concurrent shaking and incubation for 2 h at 37°C, the resultant log-phase suspension was serially diluted in sterile 0.85% NaCl solution to a concentration of 5×10^3 CFU per ml for use in the bactericidal assay.

Immunostimulants.—Three immunostimulants used for our experiments were: (a) *Vibrio* antigen (heat-killed cells of *Vibrio vulnificus*), previously shown to induce shrimp vibriosis resistance (Sung *et al.,* 1991); (b) beta-1,3-1,6-glucan extracted from *Saccharomyces cerevisiae* (Biotec Mackzymal, Tromsö), known to strengthen shrimp resistance against vibriosis (Sung *et al.,* 1994); and (c) zymosan, a beta-1,3-glucan-protein-lipid compound extracted from the cell walls of *S. cerevisiae* (Sigma), known to enhance nonspecific defenses in certain organisms via activation of the properdin system. Prior to immersion treatment, the beta-glucan and zymosan were prepared at concentrations of 1.0 mg per ml of pond water (2.5% salinity), and the concentration of the heat-killed *Vibrio* cell suspension was adjusted to 1×10^7 cells/ml.

Bacterial Clearance.—In order to determine whether or not bacteria were cleared from hemolymph, our experimental shrimp were immersed in a viable cell suspension of *V. vulnificus* (10^7 CFU/ml) for 3 h; 0.5 ml of hemolymph was drawn from each shrimp (5 individuals per test) at 5 min and 3, 6, 12, and 24 h post-immersion. One hundred μl of hemolymph diluted with sterile saline was spread onto triplicate TCBS (thiosulfate citrate bile sucrose, Difco) agar plates, which were then incubated overnight at 28°C. The numbers of bacterial colonies per plate were counted and calculated to determine the number of CFU per ml of hemolymph.

Immersion Treatment.—Three groups of tiger shrimps were separately immersed for 3 h in the suspension of beta-glucan, zymosan, and *Vibrio* antigen at a density of 4 individuals per l; control shrimp were immersed in immunostimulant-free pond water. Following treatment, the shrimps were kept in aerated pond water and 15 individuals were chosen at random at 5 min and 3, 6, 12, 24, and 72 h for hemolymph extraction.

*Anti-*E. coli *Activity Assay.*—Antibacterial activity in plasma was determined by plate count method. Approximately 15 ml of hemolymph from 15–20 shrimps were drawn into anticoagulant (0.01 M Tris-HCl, 0.25 M sucrose, 0.1 M trisodium citrate; pH 7.6) treated tubes, pooled, and centrifuged at 300 *g* for 10 min at 4°C to remove hemocytes, which were then used for the phenoloxidase activity assay. Plasma derived from the immunostimulant-treated groups or control group was concentrated by a factor of 3 via centrifugation through a centricon (Amico, Centriprep 3) at 3,000 *g* for 3 h; they were then sterilized by filtration (Sartorius filter membrane, 0.45 μm pore size). The resultant mixed suspension of 4.5 ml concentrated plasma plus 0.5 ml cell suspension of *E. coli* (5×10^3 CFU/ml) was reacted by shaking gently at 37°C for 60 min. One hundred μl of this mixture was adequately diluted and then spread onto triplicate tryptic soy agar plates, and cells of *E. coli* were allowed to grow overnight at 37°C before colony counting. The survival index (SI) of *E. coli* (modified from Wardlaw and Unkles, 1978) was calculated as follows:

$$SI = \frac{\text{CFUs per ml of mixture survived after 60-min reaction}}{\text{CFUs per ml of mixture survived at time 0 of reaction}}$$

Anti-*E. coli* activity was expressed as the ratio of the survival index for the immunostimulated shrimps to that of the control group; enhancement occurred if the ratio was less than 1.

Hemocyte Lysate Supernatant Preparation.—Shrimp hemocyte lysate supernatant (HLS) was prepared for each group as described by Smith and Söderhäll (1991). Briefly, hemocytes were washed with cacodylate-citrate buffer (0.01M sodium cacodylate, 0.45M NaCl, 0.1M trisodium citrate; pH 7.0) by centrifugation at 300 *g* for 10 min. Following removal of the supernatant, hemocyte pellets were resuspended in chilled 1:10 cacodylate (CAC) buffer (0.01 M sodium cacodylate, 0.45 M NaCl, 10 mM $CaCl_2$, 26 mM $MgCl_2$; pH 7.0). This suspension was homogenized with a sonicator (Vibra cell, AC-600) equipped with a microtip (output 5, duty cycle 50%), and centrifuged at 43,000 *g* for 20 min at 4°C. The resultant HLS was used as an enzyme source and kept at −20°C before assaying for phenoloxidase (PO) activity. HLS protein

Table 1. Clearance of viable cells of *Vibrio vulnificus* from hemolymph of tiger shrimp (*Penaeus monodon*).

After immersion	*V. vulnificus* CFU/ml (N = 5)
5 min	$(1.2 + 0.1) \times 10^6$
3 h	$(7.5 + 0.5) \times 10^4$
6 h	$(1.8 + 0.4) \times 10^2$
12 h	trace
24 h	0

CFU = Colony forming unit.
N = Number of shrimp from which hemolymph was drawn.

concentration was determined via the Bradford method, using bovine serum albumin (BioRad Protein Assay Kit II) as a standard.

Phenoloxidase Activity Assay.—PO activity was estimated with a spectrophotometer set at 490 nm, using L-dihydroxyphenylalanine (L-dopa, Sigma) as a substrate. We kept 200 μl of each sample at 37°C for 15 min, after which 400 μl of L-dopa (1.6 mg/ml in CAC buffer) was added and reacted for 1 min. Each reaction mixture was further diluted with 400 μl of CAC buffer. We then measured the absorbance of optic density at a wave length of 490 nm. One unit of enzyme activity is defined as an increase in absorbance of 0.001/min/mg protein (Söderhäll and Unestam, 1979). We used the ratio of enzyme activity of the stimulated shrimp to that of control shrimp as an index for comparing the effects of different immunostimulants on PO activity.

Intracellular Superoxide Anion (O_2^-) Assay.—This assay was conducted as described by Song and Hsieh (1994). Reactions occurred in flat-bottomed 96-well microtiter plates, with each well being coated with 100 μl poly-L-lysine solution (0.2%, Sigma) to increase the number of adhesive hemocytes (Miosky *et al.*, 1989). Approximately 1.0 ml of shrimp hemolymph was collected from a single shrimp with a 26-gauge hypodermic needle on a 2-ml syringe containing 0.4 ml of anticoagulant. The collected hemolymph was centrifuged at 300 *g* for 10 min at 4°C; the resultant hemocyte pellet was then resuspended to 10^7 cells/ml in a modified complete Hank's balanced salt solution (MCHBSS containing 10 mM $CaCl_2$, 3 mM $MgCl_2$, 5 mM $MgSO_4$, 24 mg/ml HBSS (Sigma)). One hundred μl of hemocyte suspension was added to each well (10^6 hemocytes/well) and cytocentrifuged (Kubota, KN-70) at 300 *g* for 10 min at 4°C. After removing the supernatant, 100 μl of MCHBSS was added and the hemocytes were stained with 100 μl nitroblue tetrazolium solution (NBT, 0.3% in MCHBSS) for 30 min at 37°C. The staining reaction was terminated by removing the NBT solution and adding absolute methanol. After 3 washings with 70% methanol, the hemocytes were air-dried and coated with a solution of 120 μl KOH (2 M) and 140 μl DMSO (dimethyl sulfoxide) to dissolve the cytoplasmic formazan; the optical densities of the dissolved cytoplasmic formazan were measured at 630 nm with a Precision microplate reader (Emax) (Leslie, 1987; Leslie and Allen, 1987). In order to determine the reproducibility of our results, hemocytes collected from 5 shrimps were individually assayed. We used the ratio of OD_{630} from the hemocytes of treated shrimp to the OD_{630} of the hemocytes of control shrimp as an index for comparing the effects of different immunostimulants on O_2^- generation.

Table 2. Anti-*E. coli* activity in plasma derived from tiger shrimp (*Penaeus monodon*) treated with immunostimulants via immersion.

After immersion	Survival index[a]		
	Vibrio antigen (10^7 cells/ml)	Zymosan (1 mg/ml)	Beta-glucan (1 mg/ml)
Control	0.67 (1.00)[b]	1.42 (1.00)	1.28 (1.00)
3 h	0.73 (1.08)	0.55 (0.39)	0.72 (0.56)
6 h	0.55 (0.81)	0.75 (0.53)	0.58 (0.45)
12 h	0.63 (0.94)	1.06 (0.75)	0.77 (0.60)
24 h	0.80 (1.19)	1.28 (0.90)	0.68 (0.53)

[a] Survival index (SI) = CFU after 60 min reaction/CFU at time 0 of reaction.
[b] Ratio of SI = SI of shrimp treated with immunostimulant/SI of control group.

Results

Clearance of Viable Bacteria from Plasma

To study the clearance of *Vibrio* cells from the hemolymph of our experimental tiger shrimps, we calculated the number of viable cells of *V. vulnificus* present at 5 min, 3, 6, 12, and 24 h following waterborne infection using a plate count method. It showed that about 10% of bacteria could invade hemolymph when assayed at 5 min. The number of viable *Vibrio* cells decreased substantially in a short period of time; the total *Vibrio* cell count fell by two logs within 3 h and four logs within 6 h following infection. A very small number of viable *Vibrio* cells were detected at 12 h, but were completely undetectable at 24 h (Table 1).

Anti-*E. coli* Activity of Plasma

In order to determine whether or not shrimp-clearance reaction is undertaken either via the plasma or the hemocytes, we examined the antibacterial activity of plasma, as well as intrahemocytic PO activity and O_2^- generation of shrimp in the three treatment groups. As shown in Table 2, the survival index ratio of *E. coli* was less than 1 from 3–24 h following treatment with glucan or zymosan, but less than 1 only at 6 and 12 h in shrimps treated with *Vibrio* antigen. In addition, the SI of *E. coli* in the control group was greater than 1 in both experiments that were treated by zymosan and glucan, respectively. The result showed that the two control plasma groups did not

Table 3. Intrahemocytic phenoloxidase activity in tiger shrimp (*Penaeus monodon*) treated with immunostimulants via immersion.

After immersion	Phenoloxidase activity (U/mg/min)		
	Vibrio antigen (10^7 cells/ml)	Zymosan (1 mg/ml)	Beta-glucan (1 mg/ml)
Control	5.04 (1.0)[a]	2.30 (1.0)	1.65 (1.0)
5 min	7.92 (1.6)	4.80 (2.1)	2.29 (1.4)
3 h	13.14 (2.6)	16.60 (7.2)	7.29 (4.4)
6 h	7.56 (1.5)	13.85 (6.0)	6.60 (4.0)
12 h	8.99 (1.8)	12.00 (5.2)	3.93 (2.4)
24 h	8.86 (1.8)	7.14 (3.1)	2.86 (1.7)
72 h	4.91 (1.0)	2.43 (1.1)	1.57 (1.0)

[a] Ratio of PO activity = PO activity in shrimp treated with immunostimulant/PO activity in control group.

Table 4. Intrahemocytic O_2^- production in tiger shrimp treated with immunostimulants.

After immersion	Production of O_2^- (OD_{630}) (N = 5)		
	Vibrio antigen (10^7 cells/ml)	Zymosan (1 mg/ml)	Beta-glucan (1 mg/ml)
Control	0.123 (1.00)[a]	0.114 (1.00)	0.165 (1.00)
5 min	0.094 (0.76)	0.116 (1.02)	0.173 (1.05)
3 h	0.125 (1.02)	0.210 (1.84)*	0.312 (1.89)*
6 h	0.225 (1.83)*	0.323 (2.83)*	0.272 (1.65)*
12 h	0.103 (0.84)	0.177 (1.55)	0.193 (1.17)
24 h	0.090 (0.73)	0.119 (1.04)	0.162 (0.98)

N = Sample size.
[a] Ratio of production of O_2^- = O_2^- produced by stimulated-shrimp hemocytes/O_2^- produced by control-shrimp hemocytes.
* Significant difference ($P < 0.05$) from control. The data were statistically analyzed with ANOVA designed by randomized complete block design.

exhibit anti-*E. coli* activity before stimulation.

Intracellular PO Activity

A quantitative analysis of the stimulative effects of each of the three immunostimulants on intrahemocytic PO activity in shrimp showed that PO activities from 5 min to 1 day following immersion were all greater than those observed in control shrimp; however, on day 3 the separate PO activities of the three treated shrimp dropped to those observed in control groups (Table 3). Our data on the ratio of PO activity in immunostimulated shrimp to that in control shrimp showed that intrahemocytic PO activity was increased by immunostimulant treatment immediately following immersion, with the greatest activity occurring at 3 h; in addition, we found that this activity lasted until day 3.

Intrahemocytic O_2^- Production

We also used NBT-staining to measure the generation of O_2^- by hemocytes for each of the three treatment groups. We found that O_2^- production was significantly greater in glucan- and zymosan-treated shrimp compared to control shrimp from 3–6 h following immersion. However, O_2^- production was greater in *Vibrio* antigen-treated shrimp only at 6 h (Table 4). In addition, the strongest level of O_2^- production was detected at 3 h in glucan-treated shrimp, and at 6 h in both *Vibrio* antigen- and zymosan-treated shrimp.

Discussion

Our results show that about 10% of the viable bacteria could invade the hemolymph through the immersion route. However, tiger shrimp can clear invading bacteria from their hemolymph. About 94% of cells of *V. vulniticus* were cleared within 3 h, and the escaping bacteria were almost completely cleared within the next 3 h. Viable bacterial cells were undetectable at 24 h following water-borne infection. Adams (1991) reported that more than 99% of heat-killed *Vibrio alginolyticus* were cleared from hemolymph of *Penaeus monodon* within 4 h after exposure. The difference between the experiments of Adams and our experiments might be that the clearance of viable pathogenic bacteria from hemolymph in our experiments was not as efficient as the clearance of heat-killing bacteria in the experiments of Adams. Moreover, bacteria used by Adams were completely undetectable 2–5 days following injection treatment. This delayed clearance is probably due to the fact that Adams used an EIA (enzyme immunoassay) which detected both intact bacteria and also debris resulting from either phagocytosis or digestion by lysozyme. Conclusively, clearance of foreign materials from shrimp hemolymph occurred rapidly within 6 h. Any strategy enhancing the clearance activities with an onset greater than 4 h would have a limited bactericidal effect.

Substances that have attracted the most research interest, especially in shore crabs, *Carcinus maenas* (L.), and lobsters, are bactericidins (Cornick and Stewart, 1968, 1975). However, they were not found in the plasma (Smith and Ratcliffe, 1978), but were found in the hemocytes (Chisholm and Smith, 1991). To the contrary, our re-

search showed that antibacterial activity is detectable in tiger shrimp plasma concentrated by a factor of three. Such a quick antibacterial response can be enhanced by stimulation of shrimp with the three tested immunostimulants, but it was a short-lived response. Therefore, we conjecture that antibacterial substances found in shrimp plasma may be inducibly released from hemocytes by immersing shrimp in immunostimulants. Our data also show that not all control plasma has anti-*E. coli* activity before stimulation. The possible explanation of this variation is the variability of the growth of shrimp reared together under conditions typical of shrimp ponds in Taiwan. The phenomena also occurred in PO activity (Sung *et al.,* 1994). Further research is required to understand the details of immunostimulation.

Phenoloxidase can be activated in crustaceans in vitro by LPS (lipopolysaccharide), and it serves functions in nonself recognition and host defense (Smith and Söderhäll, 1991); which include, as well, bactericidal activity and phagocytosis (Unestam and Söderhäll, 1977). Sung *et al.* (1994) have similarly reported the presence of PO in shrimp hemocytes. This enzymatic activity can be enhanced in vitro by beta-glucan treatment, which may contribute to the enhancement of disease resistance in tiger shrimp. In the present study, we confirmed that PO activity was stimulated in vivo by each of the three tested immunostimulants, and was seemingly increased before either antibacterial activity or O_2^- production. Smith and Söderhäll (1983) reported that beta-1,3 glucans can activate the proPO activating system, and initiate a cellular defense reaction, such as degranulation. Therefore, we suspect that the proPO system in shrimp is probably similar to that in other crustaceans, and may play a role in both nonself recognition and help initiate antibacterial activity and phagocytosis.

We also found that PO activity levels varied considerably according to stimulant type; the stimulative effects of beta-glucan and zymosan were stronger than that of the *Vibrio* antigen. Söderhäll and Hall (1984) reported that the proPO system in crayfish was triggered by such microbial products as LPS and beta-1,3-glucan, but through different activating pathways. Many studies showed that the activation of proPO system exerted by beta-1,3-glucan is specific via a beta-1,3-glucan binding protein (βGBP), which is found in the plasma of both insects and several crustaceans (Söderhäll *et al.,* 1994). However, in crustaceans, no specific factors have been found to bind to LPS until now. Hence, we suggest that the stimulative mechanism(s) in the proPO activating system of shrimp may differ according to the immunostimulant used, and that a βGBP-like substance may be present in shrimp plasma.

Stimulated phagocytes are known to produce highly reactive oxygen species with powerful microbicidal activity. The earliest product among highly reactive oxygen species is O_2^-. Song and Hsieh (1994) reported that O_2^- and H_2O_2 play more important roles in microbicidal activity than do OCl^- (hypochlorite) and MPO (myeloperoxidase) activity in tiger shrimp. Connors and Yoshino (1990) reported that hemocytes from *Biomphalaria glabrata* (Say) resistant to *Schistosoma mansoni* Sambon possess a greater capacity for generating superoxide anion than those taken from susceptible snails. Our results show that O_2^- production could be enhanced by treatment with the three immunostimulants. The greatest production of O_2^- occurred from 3–6 h poststimulation, and the stimulative effect lasted for no more than 24 h. Based on these results, we conclude that: (1) the phagocytotic hemocytes of shrimp could be activated to produce O_2^- and probably initiate the cascade of highly reactive oxygen species, all of which have the ability to kill invading microorganisms; (2) because of its rapid reaction rate, O_2^- was very important for acute responses; and (3) because O_2^- works nonspecifically and is thus capable of attacking host cells as well as microorganisms, a short-term response is required to confine the reaction to designated targets. Hence, work needs to be done on adequately maintaining the stimulative effects of immunostimulants for longer durations to enhance disease resistance in shrimp.

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ANNOUNCEMENT

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Case 2952. *Paraphronima crassipes* Claus, 1879 (Crustacea, Amphipoda): proposed conservation of the specific name.