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IMMUNOSTIMULATION OF TIGER SHRIMP (*Penaeus monodon*) HEMOCYTES FOR GENERATION OF MICROBICIDAL SUBSTANCES: ANALYSIS OF REACTIVE OXYGEN SPECIES

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□**Abstract**—NBT staining was used to determine the presence of superoxide anions (O_2^-) produced by tiger shrimp (*Penaeus monodon*) hemocytes attached to a coverslip. When cells were treated with β-glucan, blue granules were observed in 41% of studied hemocyte cytoplasm. For zymosan-treated, PMA-treated, and control cells, the percentages of hemocytes showing similar blue granules were 31, 9, and 5%, respectively. A comparison of stimulative effects on 15 hemocyte suspensions, each collected from a single tiger shrimp, showed that β-glucan had the strongest effect on intracellular O_2^- generation, followed by zymosan and PMA (2.5, 2, and 1.3 times greater than the O_2^- generated by the control group, respectively). After oxidizing phenol red to measure the amounts of hydrogen peroxide (H_2O_2) produced by the hemocytes, we found that β-glucan had the strongest stimulative effect (12.2 nmol/mg protein), followed by zymosan and PMA (7.2 and 2.6 nmol/mg, respectively). However, a luminol-enhanced chemiluminescence analysis of hypochlorites (OCl^-) produced by the experimental hemocytes showed that neither zymosan nor β-glucan had a stimulative effect on OCl^- production. However, following PMA stimulation, hemocyte chemiluminescence was detected although only at 1.7 mV. Using H_2O_2 as substrate and guaiacol as an electron acceptor, the enzyme activity of crude enzyme extract derived from broken hemocytes was analyzed; enzyme activity similar to that of human myeloperoxidase (MPO) (0.104 U/mg protein) was observed. The data showed that only PMA had any stimulative effect on MPO-like enzyme activity (2.23 times that of the control group); zymosan and β-glucan did not have any observable effects on this specific enzyme activity. This is the first documented demonstration of a respiratory burst in shrimp hemocytes.

□**Keywords**—Immunostimulant; β-glucan; PMA; Zymosan; Tiger shrimp (*Penaeus monodon*); Hemocytes; Reactive oxygen species.

Introduction

Polymorphonuclear leucocytes and monocytes have been proven to be the main hemocytes in vertebrate phagocytosis. When microorganisms are engulfed by these hemocytes, a series of microbicidal substances that either inhibit microbial activities or completely digest the microorganisms is generated. These substances include highly reactive oxygen species such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), hydroxide ions (OH^-), singlet oxygen (O_2^1), myeloperoxidase (MPO)-catalyzed hypochlorite, and digestive enzymes within cytoplasmic granules.

Numerous assays can be used to detect these highly reactive oxygen spe-

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cies. Nitroblue tetrazolium (NBT) stain-
ing has been used for both qualitative
and quantitative analyses of O_2^- gener-
ated by hemocytes, and the oxidation of
phenol red has been used as an indicator
for quantifying hemocyte-generated ex-
tracellular H_2O_2 (1); in addition, luminol-
enhanced chemiluminescence has been
used to analyze OCl^- (2). However, few
investigators have studied the metabolic
events associated with phagocytosis in
shrimp or the activation of shrimp
phagocytosis by foreign materials.

For the present study, the reactive ox-
ygen species and MPO generated by ti-
ger shrimp (*Penaeus monodon*) hemo-
cytes were analyzed via the above-
mentioned methods. In addition, the
effects of immunostimulants such as
phorbol myristate acetate (PMA), zymo-
san, and β-glucan on the generation of
reactive oxygen species by shrimp
hemocytes were compared. Our results
may lead to the creation of an effective
strategy for enhancing disease resistance
in cultured shrimp.

Materials and Methods

Hemocytes

Tiger shrimp (purchased from a local
market) weighing 25–40 g were kept in a
120-L fiber reenforced plastic container
supplied with a constant flow of recircu-
lating brackish water with 2.5% salinity
and 25°C temperature. Approximately
1.5 mL of shrimp hemolymph was col-
lected from the first abdominal segment
of a single shrimp with a 26-gauge hypo-
dermic needle on a 2-mL syringe con-
taining 0.5 mL of anticoagulant (0.01 M
tris-HCl, 0.25 M sucrose, 0.1 M sodium
citrate; pH 7.6). The collected he-
molymph 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 bal-
anced salt solution minus phenol red
(MCHBSS, Sigma).

Immunostimulants

We tested the following immunostim-
ulants for the generation of various reac-
tive oxygen species from shrimp hemo-
cytes: (a) phorbol 12-myristate 13-
acetate (PMA, Sigma); (b) zymosan (a
protein–carbohydrate complex ex-
tracted from the cell walls of *Saccharo-
myces cerevisiae*, Sigma); (c) β-glucan
(β-1,3, and β-1,6 linked polyglucose) ex-
tracted from *S. cerevisiae* (Biotec Mack-
zymal, Tromso).

PMA is a surface-active agent of re-
spiratory burst response in fish (3). Its
soluble nature increases the potential of
achieving reproducible results from su-
peroxide anion assays (4). Zymosan
(used as a possible natural immunostim-
ulant) is known to enhance nonspecific
defenses in certain organisms via the ac-
tivation of the properdin system. β-glu-
can (also used as a natural immunostim-
ulant) has been shown to induce cellular
defense responses in crustaceans (5,6).
Our preliminary research showed that
the optimal O_2^- stimulative concentra-
tions of PMA, zymosan, and β-glucan
were 250 ng/mL, 2.5 mg/mL, and 2 mg/
mL, respectively.

Intracellular Superoxide Anion
(O_2^-) Detection

Qualitative assay. Fifty microliters of
shrimp hemolymph was placed on a cov-
erslip. After 1 h of adhesion, nonadhe-
sive cells were washed off with MCH-
BSS. The hemocyte-containing coverslip
was then turned upside down onto a slide
that had been previously coated with a
mixture of 50 μL NBT (0.3% in MCH-
BSS) and an equal volume of one of the

three immunostimulants. After 30 min at 37°C, the NBT reduction reaction had taken place and individual hemocyte cytoplasms examined under a light microscope for blue granules of reduced formazan. We then calculated the percentage of hemocytes showing these cytoplasmic blue granules from 250 observed hemocytes. Hemocytes that reacted with the NBT/MCHBSS mixture (instead of the NBT/immunostimulant mixture) were used as control.

Quantitative assay. Each well of a 96-well microtiter plate was coated with 100 μL of poly-L-lysine solution (0.2%, Sigma) to increase the number of adhesive hemocytes (7). One hundred microliters of hemocyte suspension collected from a single shrimp was added to each of the four wells and then cytocentrifuged (Kubota, KN-70) at $300 \times g$ for 10 min. After removing the supernatant, 100 μL of each immunostimulant (PMA, zymosan, or β-glucan) was added and allowed to react for 30 min at 37°C. MCHBSS was added to the remaining hemocyte suspension as a control. We then discarded the immunostimulant solution, washed the hemocytes three times with MCHBSS, then stained them with NBT solution (0.3%, 100 μL) for 30 min at 37°C. The staining reaction was terminated by removing the NBT solution and adding absolute methanol. After three washings with 70% methanol, the hemocytes were air-dried and coated with a solution of KOH (120 μL, 2 M) and DMSO (140 μL) to dissolve the cytoplasmic formazan; the optical densities of the dissolved cytoplasmic formazan at 630 nm (OD_{630}) were then measured with a Precision microplate reader (Emax) (8,9). In order to determine the reproducibility of our results, hemocytes collected from 15 shrimp were individually assayed. We used the ratio of OD_{630} from the stimulated hemocytes to the OD_{630} of control hemocytes as an index for comparing the effects of different immunostimulants on the generation of O_2^-.

Extracellular Hydrogen Peroxide (H_2O_2) Detection

Genfa and Dasgupta (10) first described an assay of the peroxidase-dependent oxidation of phenol red by H_2O_2. Following their procedures, we prepared a phenol red solution (PRS) containing 0.001% phenol red and 0.005% horseradish peroxidase just before use. Approximately 10^7 hemocytes were placed onto a 24-well microtiter plate precoated with poly-L-lysine. The hemocytes were first washed three times with MCHBSS, then 1 mL of PRS and 0.5 mL of one of the three immunostimulants were added to each well to stimulate the respiratory burst. After being allowed to react for 1 h at 37°C, optical densities were determined with a spectrophotometer (Hitachi 2000) at 610 nm using PRS plus MCHBSS as a blank.

We converted our readings to nanomoles of H_2O_2 by using a standard curve established from solutions with known H_2O_2 molarities (2, 4, 6, 8, 10 μM). Protein concentrations of NaOH-dissolving hemocytes present in each well were estimated with a Bradford reagent (Bio-Rad); we then calculated the H_2O_2 (nmol) released per hemocyte protein (mg) for each well.

Chemiluminescence (CL) Assay

According to procedures described by Van Dyke et al. (2), 0.3 mL of luminol solution (0.1 mM, Sigma) and an equal volume of hemocyte suspension (5×10^7 cells/mL) were mixed and maintained at 24°C. This solution was then put into a glass tube (8-mm diameter $\times$ 50-mm height) that was placed into a reaction chamber to measure background light.

After establishing constant background counts, 0.3 ml of each immunostimulant suspension was added to initiate CL; CL values were measured for 1 h at 1-min intervals with a luminometer (Turner, KD-20e).

MPO Activity Assay

Crude MPO extraction. Our MPO extraction technique was a modification of the procedures described by Bakkenist et al. (11) and Pember et al. (12,13). Hemocytes were suspended at 10^7 cells/ mL in 0.34 M ice-cold sucrose containing 10 μg/mL each of phenylmethylsulfonylfluoride (PMSF) and L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK) plus 1 mM EDTA. Homogenates were produced with a Teflon-glass homogenizer (4000 rpm, 1 min, Tline), then centrifuged at 500 × g for 5 min at 4°C. Supernatants were collected, and pellets containing unbroken hemocytes were rehomogenized and centrifuged, after which the supernatants were pooled; this process was repeated (usually 1–2 times) until MPO was essentially undetectable. The pooled supernatants were centrifuged at 27000 × g for 15 min at 4°C; supernatant containing MPO was dialyzed against 10 mM sodium phosphate buffer (pH 7.0), and the resultant precipitate was removed by centrifugation at 27000 × g for 15 min.

Assay. Four hundred microliters of each of the three immunostimulants was added to the hemocyte suspension (600 μL) and allowed to react at 37°C for 40 min. An equal volume of MCHBSS added to a hemocyte suspension was used as a control. Crude MPO was extracted using the procedures described above, and enzyme activity was measured using methods described by Desser et al. (14). One unit of activity was defined as the amount of enzyme catalyzing the decomposition of 1 μmol

H_2O_2 in 1 min at 25°C, with a 26.6 mM^{-1} cm^{-1} coefficient for tetraguaiacol at 470 nm. Protein concentrations of crude MPO were determined via a Bradford procedure, with bovine serum albumin used as a standard. Our preliminary investigations revealed the following optimal values for the measurement of MPO activity: substrate solution pH at 7.0; H_2O_2 concentration at 0.4 mM; and NaCl concentration at 2.0%.

Results

We found that most of the shrimp hemocytes that adhered to our coverslips had both a low ratio of nucleus size to cytoplasm as well as a low number of small granules in their cytoplasm. We used NBT staining to determine the presence of O_2^- anion produced by these hemocytes. In β-glucan-treated cells, blue granules were observed in the cytoplasm of 41% of the hemocytes (Fig. 1a). For zymosan-treated, PMA-treated, and control cells, the percentages of hemocytes with blue granules present were 31, 9, and 5%, respectively (Figs. 1b–d).

A quantitative analysis of the stimulative effects of the three immunostimulants on hemocytes revealed that β-glucan had the strongest effect, followed by zymosan and PMA (2.5, 2, and 1.3 times stronger than that of the control group, respectively) (Table 1). After oxidizing phenol red to measure the amount of H_2O_2 produced by our observed hemocytes, we found that β-glucan had the strongest stimulative effect (12.2 nmol/ mg protein), followed by zymosan and PMA (7.2 and 2.6 nmol/mg, respectively) (Fig. 2). However, the amount of H_2O_2 produced by the stimulated hemocytes varied significantly among individual shrimp. Luminol-enhanced chemiluminescence analysis of the OCl$^-$ produced by our experimental hemocytes showed that zymosan and β-glucan had no stimulative effect on OCl$^-$ production; PMA
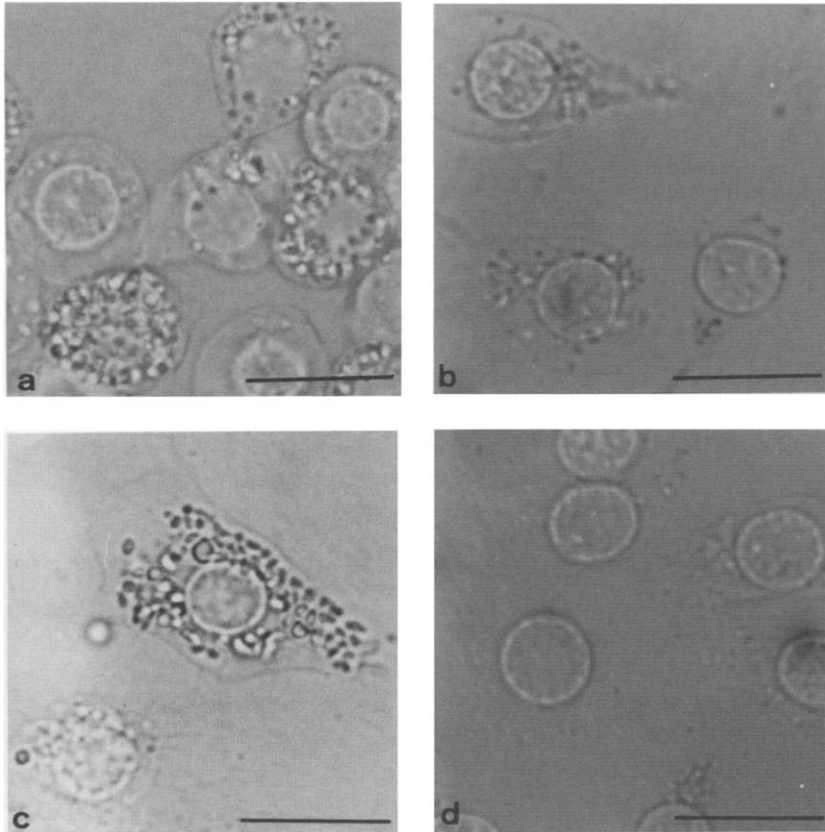
Figure 1. Shrimp hemocytes stained with nitroblue tetrazolium (0.3%). Blue granules were observed in the cytoplasm of 41% of the hemocytes treated with (a) β-glucan (2 mg/mL). For (b) zymosan (2.5 mg/mL) treated, (c) phorbol myristate acetate (250 ng/mL) treated, and (d) MCHBSS treated cells, the percentages of hemocytes with blue granules present were 31, 9, and 5%, respectively. ×2500; bar = 10 μm.

stimulation enhanced hemocyte chemiluminescence, but only to a maximum of 1.7 mV (Fig. 3).

Finally, using H_2O_2 as substrate and guaiacol as an electron acceptor, we analyzed the enzyme activity of crude extract derived from broken hemocytes, and found enzyme activity similar to that of human MPO (0.104 U/mg protein). Our data showed that only PMA had any stimulative effect on MPO-like enzyme activity (2.23 times that of the control

Table 1. Effects of Immunostimulants on Production of O_2^- by Hemocytes Assayed Via Staining With NBT.

Immunostimulants	Concentration	Average Relative Production of O_2^- ($n = 15$)
MCHBSS	—	1.000 ± 0
PMA	250 ng/mL	1.296 ± 0.249
Zymosan	2.5 mg/mL	2.014 ± 0.276
β-glucan	2.0 mg/mL	2.525 ± 0.504

Relative production of $O_2^- = OD_{630}$ of stimulated hemocytes/OD_{630} of control hemocytes.
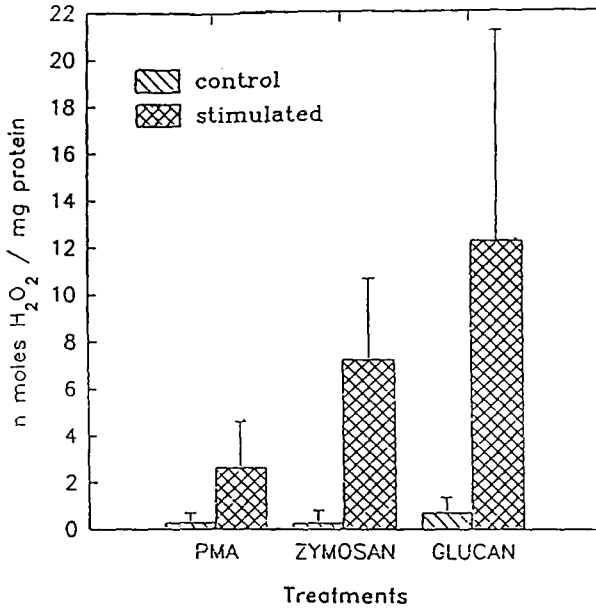
Figure 2. Effects of different immunostimulants on the production of H_2O_2 by hemocytes assayed with oxidized phenol red. PMA: 250 ng/mL; zymosan: 2.5 mg/mL; β-glucan: 2 mg/mL. Sample sizes, PMA: 21; zymosan: 20; β-glucan: 23.

group); zymosan and β-glucan did not have any observable effects on this activity (Table 2).

Discussion

NBT staining was used to evaluate the effects of three immunostimulants, that is, for comparing both the amount of cytoplasmic blue granules and the percentage of hemocytes showing blue granules. Only 41% of β-glucan-treated hemocytes showed blue granules in their cytoplasm; more than half of the observed hemocytes remained inactive, showing that the adhesive hemocytes, mostly small granular cells, apparently did not undergo the respiratory burst. Hence, we believe that functionally different subpopulations probably exist among small granular hemocytes, showing the need for the further classification and separation of hemocytes.

Because O_2^- is the first product released from a respiratory burst, O_2^- measurement has been accepted as an accu-

rate method for quantifying the intensity of a respiratory burst (1). A quantitative comparison of the stimulative effects of PMA and zymosan on shrimp hemocytes, as detected by the reduction of NBT, revealed that the amounts of intracellular O_2^- produced by shrimp hemocytes were 1.3 and 2.0 times that of the control group, respectively. A similar result (1.2 times greater) was obtained from isolated rainbow trout macrophages that had been stimulated with PMA (15). However, the amounts of O_2^- released by human neutrophils were approximately 9 and 8 times normal levels following stimulation with either PMA or opsonized zymosan, respectively (16).

In addition, the stimulative effects of PMA and zymosan on shrimp hemocytes, as detected via the oxidation of phenol red, showed that amounts of released H_2O_2 were 17.9 and 25.8 times that of the control group, respectively. Meanwhile, the amount of H_2O_2 released by isolated rainbow trout macrophages treated with PMA was three times greater than normal (15). In addition, the
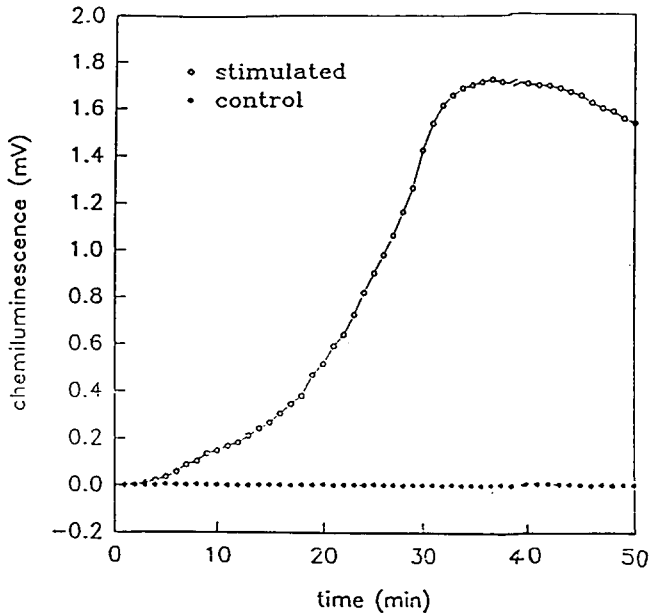
Figure 3. Chemiluminescence induced by OCl^- anions produced by hemocytes treated with PMA (500 ng/mL) at 24°C. Hemocyte concentration: 5×10^7 cells/mL; luminol concentration: 0.1 mM.

amounts of H_2O_2 released by human macrophages following stimulation with PMA or opsonized zymosan were 120 and 23 times normal levels, respectively (17). Together, these results demonstrate that tiger shrimp responses to immunostimulants and the amounts of microbicidal substances generated by shrimp hemocytes were similar to those observed in fish macrophages, but generally lower than those observed in human monocytes and neutrophils.

One probable explanation for this difference is that the structure or number of receptors on the hemocyte membranes

of shrimp, fish, and humans differ significantly. Oxygen metabolites released by phagocytes are highly reactive; it is possible that they play both beneficial and detrimental roles in chronic inflammation, and therefore the number of metabolites released by the phagocytes should be strictly regulated. As previously noted, luminol-enhanced chemiluminescence analysis showed that OCl^- was not detectable in our observed stimulated shrimp hemocytes during the phagocytic process, probably because associated enzyme activity in the hemocytes was very low. Using H_2O_2 as a sub-

Table 2. Effects of Immunostimulants on Activity of Myeloperoxidase Extracted From Shrimp Hemocytes Assayed Via Desser's Method.

Immunostimulants	Concentration	Average Relative MPO Activity ($n = 10$)
MCHBSS	—	1.000 ± 0
PMA	500 ng/mL	2.230 ± 0.729
Zymosan	2 mg/mL	1.141 ± 0.180
β-glucan	2 mg/mL	0.972 ± 0.138

Relative MPO activity = MPO activity of stimulated hemocyes/MPO activity of control hemocytes.

strate and guaiacol as an electron acceptor, we observed enzyme activity similar to that of human MPO; however, the enzyme activity of crude extract derived from broken hemocytes was much lower than that observed in human polymorphonuclear (PMN) leucocytes, probably because the shrimp MPO in our experiment was extracted from whole hemocytes instead of phagocytic cells (as in the case for studies involving human cells). The lack of detection of OCl^- was further confirmed by the low rate of MPO enzyme activity observed in shrimp.

In conclusion, β-glucan had the strongest stimulative effect on hemocytes in terms of generating O_2^- and H_2O_2, but PMA had the strongest stimulative effect on OCl^- production and MPO activity, thus underscoring the fact that different immunostimulants affect different stages of the phagocytic process. Similar to microbicidal activity in human PMN, phagocytosis is correlated with the production of reactive oxygen species (18). It appears that O_2^- and H_2O_2 play more important roles in tiger shrimp microbicidal activity when compared to OCl^- and MPO activity; therefore, we suggest that treatment with β-glucan can enhance nonspecific disease resistance in shrimp. In addition, this nonspecific disease resistance may also account for previous observations of enhanced growth rates in tiger shrimp induced by treatment with β-glucan (19).

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