SUISANZOSHOKU 49(3), 389−396 (2001)

Participation of Reactive Oxygen Intermediates in the Killing of Ingested Bacteria by *Crassostrea gigas* Hemocytes

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(Accepted August 21, 2001)

Abstract: The participation of reactive oxygen intermediates (ROIs) in the killing of ingested bacteria by hemocytes of the Pacific oyster, *Crassostrea gigas*, has been investigated under aerobic and anaerobic conditions. In the present study, we used five different species as target organisms, i.e., *Arthrobacter ramosus*, *Bacillus subtilis*, *Deleya pacifica*, *Escherichia coli*, and *Micrococcus luteus*. In order to measure the ROI-production by the *C. gigas* hemocytes, we used chemiluminescence (CL). Significant stimulation of the hemocyte-derived CL occurred in response to *A. ramosus*, whereas no increase in the ROI-generation was observed when the *C. gigas* hemocytes were exposed to the other four bacterial species. Although the *C. gigas* hemocytes exerted a phagocytic ability against all the bacterial strains tested, there were no statistical differences in all the bacterial strains under both aerobic and anaerobic conditions. In the anaerobic environment, the intracellular killing percent of *A. ramosus* by the *C. gigas* hemocytes significantly decreased to 36.7% when compared with the 66.2% intracellular killing under the aerobic conditions. Contrary to the intracellular killing activity of the *C. gigas* hemocytes toward the other four bacterial strains, there was no significant difference between the aerobic and anaerobic environments. Intracellular killing of *A. ramosus* was also repressed by the addition of diphenyleneiodonium (DPI), which is a potent inhibitor of NADPH oxidase.

Key words: *Crassostrea gigas*; Hemocyte; Phagocytosis; Intracellular killing

The host defense system of bivalve mollusks is considered to mainly depend on circulating hemocytes (present in the hemolymph), which possess a strong migration ability in response to invading microorganisms and subsequently actively phagocytize these invaders[1]. The hemocytes of mollusks morphologically resemble mammalian phagocytic leukocytes[2,3] and, like these leukocytes, have the ability to recognize, engulf, and internally degrade foreign microorganisms[1,4].

Phagocytosis by mammalian macrophages and neutrophils activates a membrane-bound oxidase, and is accompanied by an increase in oxygen uptake (the 'respiratory burst'). In this respiratory burst process, the initial biochemical event during the reactive oxygen intermediate (ROI) production is the conversion of molecular oxygen (O_2) to superoxide anion (O_2^-) catalyzed by the NADPH oxidase system[5].

Many investigations have demonstrated the generation of ROIs, such as O_2^- and hydrogen peroxide (H_2O_2), by stimulated hemocytes in several bivalve species[6]. The oxidative activity in molluscan hemocytes has been shown to be valuable in defining host-defensive events[4] and hemocyte-pathogen interactive modulation[6].

Therefore, the following hypothesis has been developed. Molluscan phagocytic hemocytes possess a specific ROI-forming system associated with NADPH oxidase, which becomes functional due to stimulation mediated by the phagocytosis of foreign particles and microbes, and the ROIs contribute to the bactericidal action of the hemocytes, which is similar to the professional phagocytic leukocytes in mammals[4]. However, in serial investigations performed by Bramble and Anderson[7,8], *Crassostrea virginica*

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hemocytes stimulated by the bacterium *Listonella* (formerly *Vibrio*) *anguillarum* produced low levels of ROIs, which may be incapable of surpassing the bacterial antioxidant capability. Moreover, Bramble and Anderson[9] demonstrated that *C. virginica* hemocytes did not generate ROIs upon stimulation with two bacterial species, whereas the bactericidal activity of the *C. virginica* hemocytes against these bacteria was clearly evident. These results suggested that ROIs generated by *C. virginica* hemocytes do not participate in the killing of these three species of bacteria as bactericidal effectors in the host-defense system of oyster hemocytes. In the hemocytes of mollusks, however, some of the correlated details between the ROI generation and intracellular bactericidal ability have yet to be unequivocally demonstrated.

The objective of this study is to present the question of whether *C. gigas* hemocyte-generated ROIs contribute to the intracellular killing of bacteria engulfed by the hemocytes. We examined the phagocytic ROI-response of the *C. gigas* hemocytes to these bacteria using the chemiluminescence (CL) reaction. Furthermore, in order to determine whether these bacteria are killed and degraded by the *C. gigas* hemocytes in the absence of O_2, we ran *in vitro* experiments of phagocytosis and intracellular killing using an anaerobic environment. We also examined the effects of diphenyleneiodonium (DPI), a potent NADPH oxidase inhibitor, on the intracellular killing activity of the *C. gigas* hemocytes under aerobic and anaerobic conditions.

Materials and Methods

Chemicals

Phenyl-10-methyl-acridinium-9-carboxylate fluorosulfonate (PMAC), which has a 100-fold higher sensitivity than luminol and lucigenin for the detection of O_2^- generated by the *C. gigas* hemocytes[10] and also has a high specificity for the detection of H_2O_2 (obtained from the product data from Dojindo Laboratories), was purchased from Dojindo Laboratories, Kumamoto, Japan. PMAC was prepared on the day of use in a balanced salt solution for the *C. gigas* hemocytes[11] (oyster BSS: NaCl, 446.6 mM; KCl, 14.5 mM; $CaCl_2$, 8.6 mM; $MgCl_2$, 10.6 mM; $MgSO_4$, 14.2 mM; $NaHCO_3$, 3.0 mM; NaH_2PO_4, 0.08 mM; glucose, 5.6 mM; pH 7.8) at an initial concentration of 240 μM.

Diphenyleneiodonium (DPI) was used for inhibition of the NADPH oxidase activities in the hemocytes of *C. virginica*[9] and *C. gigas*[11]. DPI chloride (Dojindo Laboratories) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 1.6 mM and maintained at −80℃ (stock solution); the working solution was prepared in oyster BSS. The stock solution of diethylenetriamine-pentaacetic acid (DTPA, Dojindo Laboratories) was prepared in sterile distilled water (SDW) at a concentration of 10 mM and stored at 4℃. Microbiological media were purchased from Difco Laboratories, Detroit, MI, USA, and prepared according to the manufacturer's instructions. All other reagents were purchased from Wako Pure Chemicals Industries, Osaka, Japan, and were the highest analytical grade commercially available.

Oysters

The specimens of the Pacific oyster, *C. gigas*, with an average shell height of 13.7 cm used during this study were obtained from a hanging-cultured bed in Matsushima Bay, Miyagi Prefecture. They were then transferred to our laboratory and held in 150 *L* aquariums with recirculated, filtered artificial seawater (MARINE ART BR, Senju Seiyaku Co., Japan) for 2-5 days. The water temperature was maintained at 15± 1℃.

Isolation of C. gigas hemocytes

Hemolymph was withdrawn from the blood sinus in the adductor muscle using a tuberculin syringe and 23 gauge, 1.5 inch needle. Each *C. gigas* was bled once only. The hemolymph was transferred to a 15 *mL* centrifuge tube and centrifuged at 290×*g* for 20 min at 4℃ to separate the hemocytes. The resulting hemocyte pellet was washed three times with oyster BSS. The washed hemocyte pellet was then resuspended to 1×10^6 cells/*mL* in oyster BSS. More than 90% of the hemocytes were viable based on the

dye exclusion test with 0.05% trypan blue in oyster BSS. To minimize cell clumping, the hemocyte suspension was maintained on ice until use.

Bacterial strains

Five species of bacteria were examined in this study. Based on gram-coloration, three positive species were used, i.e., *Arthrobacter ramosus* (strain IAM 12344), *Bacillus subtilis* (strain IFO 13719), and *Micrococcus luteus* (strain ATCC 4698). Two gram-negative species were also used, i.e., *Deleya pacifica* (strain IAM 12553) and *Escherichia coli* (strain IAM 1264). It is well documented by some investigations that many bacterial species possess defense systems against oxidative stress, including hemocyte (or leukocyte)-generated ROIs. Moreover, Elsbach *et al.*[12] reported that the susceptibilities to the bactericidal effects of mammalian leukocytes widely differ between interspecies of bacteria. Therefore, we used in the present study five different species as target organisms. They are environmental and avirulent bacteria for oysters. The strain of *A. ramosus* was catalase negative, whereas the other four species had catalase activity. Moreover, the *M. luteus* strain possessed a large amount of carotenoid pigments, which showed a strong antioxidant activity[13]. The three IAM strains were kindly supplied from the Institute of Molecular and Cellular Biosciences, the University of Tokyo, Tokyo, Japan. The IFO strain was purchased from the Institute for Fermentation, Osaka, Japan. The ATCC strain was obtained from the American Type Culture Collection, Rockville, MD, USA.

Bacterial cultivation

The *D. pacifica* strain was cultured in marine broth 2216 at room temperature for 48 h. The grown bacteria were centrifuged at 2,450×*g* for 20 min, washed three times with oyster BSS and resuspended in oyster BSS. The other four strains were grown in nutrient broth at room temperature for 24-48 h. Bacterial cells were harvested by centrifugation, washed three times with 0.01 M phosphate-buffered 0.15 M saline (PBS, pH 7.4), and resuspended in oyster BSS. The final concentrations of the bacterial strains were approximately 10^7 colony forming units (CFU) /*mL*.

Chemiluminescent response of C. gigas hemocytes to the five bacterial strains in an aerobic environment

C. gigas hemocytes (5×10^5 cells) suspended in oyster BSS were placed in each well of a 24-well cell culture plate, which was then incubated for 30 min to allow for cell adherence. Following the incubation, the overlaying BSS was removed from each well, and each monolayer of the hemocytes was then washed twice with oyster BSS. Each of the hemocyte monolayers was overlaid with a reaction mixture for the chemiluminescence (CL) procedure. The reaction mixture contained PMAC (final concentration of 2.4 μM), DTPA (final concentration of 500 μM) and oyster BSS in a total volume of 1.9 *mL*. The CL reaction was initiated by the addition of 100 μ*L* of bacterial suspension (5×10^7 CFU/*mL*). Each bacteria was present at a target to hemocyte ratio of 100:1. A background CL generated by unstimulated hemocytes (a negative control) was measured by the addition of oyster BSS instead of the bacterial suspension. Bramble and Anderson[7] reported that viable bacterial species, which were used to stimulate the CL response of the *C. virginica* hemocytes, generated lucigenin CL. In this study, therefore, the CL of each bacterial species without hemocytes was also quantified as a bacterial CL. An ARGUS-50 2D Lumino-Imageprocessor (Hamamatsu Photonics., Hamamatsu, Japan) was used to detect the PMAC-enhanced CL activity. The CL was quantified for 60 min and a 0.5 min counting interval at room temperature. The CL response was expressed as the total photon counts in each well for 60 min.

Phagocytosis assay in aerobic and anaerobic environments

In order to measure the *in vitro* phagocytosis of the five species of bacteria by the *C. gigas* hemocytes in an anaerobic environment, we performed the aerobic and anaerobic phagocytosis assay according to Hampton and Winterbourn[14]. This assay was performed in

chambers, through which a mixture of 10% carbon dioxide (CO_2) and 90% nitrogen (N_2) gas was passed at a flow of 50 *mL*/min for 10 min. Oyster BSS was saturated with the mixture of 10% CO_2 and 90% N_2 filtered through a sterile membrane filter (Millex FG50, 0.2μm pore, Nihon Millipore, Ltd., Tokyo, Japan) before preparation of the hemocyte and bacterial suspensions (preparation of anaerobic oyster BSS). Drops of 100μL of hemocyte suspension (5×10^6 cells/*mL*) were mounted onto multiwell assay slides (AR BROWN Co., UK) in the mixture of 10% CO_2 and 90% N_2-saturated chambers sealed with air-tight silicon stoppers and were incubated for 30 min at 20℃. After incubation, the hemocyte monolayers were prepared, and washed twice with anaerobic oyster BSS. The monolayers were overlaid with 100-μL drops of the bacterial suspension (5×10^8 CFU/*mL*) and incubated for another 30 min at 20℃. At the end of the incubation period, the excess bacteria were removed by thoroughly washing the monolayers with anaerobic oyster BSS. The hemocyte-bacteria monolayers were fixed in absolute methanol, and stained with May-Grüwald and Giemsa stain. The extent of the phagocytosis was determined by randomly counting a combined total of 500 phagocytic and nonphagocytic hemocytes from each monolayer under a light microscope. After observing 500 hemocytes, the phagocytic percentage and phagocytic index were calculated. The phagocytic percentage denotes the percentage of phagocytosis-positive cells, and the phagocytic index is expressed as the average number of bacterial cells ingested in one hemocyte.

The aerobic chambers were bubbled with air at approximately the same rate as the mixture of 10% CO_2 and 90% N_2 in entered the anaerobic chambers.

Intracellular killing assay in aerobic and anaerobic environments

The hemocytes phagocytizing bacteria were further incubated in air (aerobically) or in the mixture of 10% CO_2 and 90% N_2 (anaerobically) for 60 min at 20℃. After stopping the reaction, the hemocytes were disrupted with Triton X-100 (final concentration of 0.1%) in oyster BSS (500μL). The hemocyte suspension was transferred to a test tube and diluted in 9.5 *mL* of ice-cold 0.85% NaCl. The tube was vigorously voltexed to lyse the hemocytes, and viable intracellular bacteria were released into the suspension. The viable number of phagocytized bacteria was determined at 0 and 60 min after incubation by counting the colonies formed on agar plates. The percent exhibiting the intracellular killing (killing rate) was calculated by the following formula[15]:

killing rate (%) = (1 − CFU after 60 min/CFU at 0 min) × 100.

Effect of DPI as an NADPH oxidase inhibitor on intracellular killing of A. ramosus and M. luteus by C. gigas hemocytes in an aerobic environment

The hemocyte monolayers were also prepared as described in the phagocytosis assay section. *Arthrobacter ramosus* and *M. luteus* were used as foreign organisms to determine the inhibitory effects of DPI on the phagocytosis and intracellular killing by the *C. gigas* hemocytes. For the DPI treatment, bacteria were suspended in the DPI solution at the final concentration of 1.6μM.

Statistical analysis

The experiments were conducted in triplicate of five specimens each. Fisher's least difference (Fisher's LSD) method was used to test whether the results of the aerobic and anaerobic hemocytes groups were significantly different at the 5% level ($p<0.05$). Fisher's LSD was also used for the analyses of the phagocytic ability and intracellular killing activity of the hemocytes in the presence and absence of DPI. Data for the CL responses were assessed using a one-way analysis of variance (ANOVA). If significant differences were found ($p<0.05$), a Dunnett multiple comparison test (Dunnett's test) was conducted using the CL response of the unstimulated hemocytes (hemocytes only) as the negative control, to which those of the hemocytes stimulated by the addition of bacteria were compared. The results are presented as the mean ± standard error of the mean (SEM).

Results

Chemiluminescent response of C. gigas hemocytes to the five bacterial strains in an aerobic environment

As indicated in Fig. 1, the viable cells of all the tested bacteria generated PMAC CL (each of the BO columns in Fig. 1). Because unstimulated hemocytes also produced PMAC CL, it was included as a background control (the HO column in Fig. 1). The hemocyte-derived PMAC CL was significantly stimulated by only *A. ramosus* of the five tested bacterial strains. The CL generated by the hemocytes in response to *A. ramosus* was 11 times higher compared with that produced by the unstimulated hemocytes (the background CL). However, the CL generated by the live bacteria of the four strains except for *A. ramosus* was almost equal to the CL produced by the hemocytes in response to the bacteria.

Comparison of phagocytosis of each bacterial strain between aerobic and anaerobic environments

C. gigas hemocytes exerted phagocytic ability against all the bacterial strains tested. The phagocytic percentages varied with the bacteria and ranged from 13.3% to 47.6% (Table 1). There were no statistical differences in the phagocytic percentages for all the bacterial strains between the aerobic and anaerobic environments. Similarly, the phagocytic index also varied among the five bacterial species ranging from 1.6 to 21.0. However, the anaerobic condition did not affect the *C. gigas* hemocyte phagocytic indices against all the bacterial strains tested.

Effects of an anaerobic environment on the intracellular killing activity of bacteria by the C. gigas hemocytes

The *C. gigas* hemocytes exerted intracellular killing activity against all the bacterial strains tested. More than 40% of the phagocytized bacterial cells were killed by the *C. gigas* hemocytes in the aerobic environment (Table 2). In the anaerobic environment, however, the per-

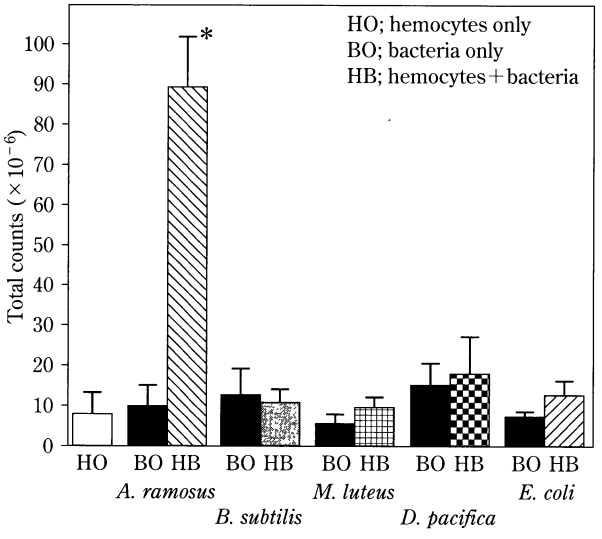

Fig. 1. *Crassostrea gigas* hemocyte PMAC-evoked chemiluminescence (CL) in response to five tested species of bacteria. Results are expressed as means±SEM of total photon counts of CL for 60 min (n=5). HO, background CL associated with 5×10^5 hemocytes without bacteria. BO, background CL associated with 5×10^7 CFU of each bacterial species without hemocytes. HB, CL associated with both hemocytes (5×10^5 cells) and each of bacteria (5×10^7 CFU). *Significantly greater than the HO count (Dunnett's test, $p<0.05$).

Table 1. Phagocytic percentage*1 and phagocytic index*2 of each bacterium by *C. gigas* hemocytes in the aerobic and anaerobic environments

Bacteria	Phagocytic percentage		Phagocytic index	
	Aerobic	Anaerobic	Aerobic	Anaerobic
A. ramosus	18.3±4.5	18.0±6.1	3.4±2.0	3.9±2.3
B. subtilis	40.9±8.6	42.4±5.5	15.2±6.7	14.4±7.2
D. pacifica	13.5±2.8	13.3±3.2	1.6±1.2	1.7±1.0
E. coli	45.9±9.1	47.6±6.7	10.3±4.1	10.8±4.9
M. luteus	34.6±7.0	33.8±4.7	20.4±6.4	21.0±7.4

*1Phagocytic percentage denotes the percentage of phagosytosis-positive cells.

*2Phagocytic index is expressed as the average number of bacterial cells ingested in one hemocyte. Each result shows the mean±SEM (n=5). There were no significant differences in the phagocytic percentage and phagocytic index of the *C. gigas* hemocytes toward each bacterial species in both the aerobic and anaerobic environments ($p<0.05$).

Table 2. Intracellular killing rate*1 of each bacterium by *C. gigas* hemocytes in aerobic and anaerobic environments

Bacteria	Killing rate (%)	
	Aerobic	Anaerobic
A. ramosus	66.2±9.4[a]*2	36.7±8.3[b]
B. subtilis	42.4±6.1	44.1±8.9
D. pacifica	77.9±5.4	80.2±7.2
E. coli	54.6±11.3	57.9±8.3
M. luteus	48.3±8.6	46.7±9.1

*1Killing rate was calculated by the following formula:

$$1-\frac{\text{CFU after 60 min}}{\text{CFU at 0 min}}\times100$$

*2The data having different alphabetical designations are significantly different using Fisher's LSD method ($p<0.05$; n=5).

cent intracellular killing of *A. ramosus* significantly decreased to 36.7% when compared with the 66.2% intracellular killing under the aerobic condition. There was no significant difference in the other four bacterial strains between the aerobic and anaerobic environments. Neither the aerobic nor the anaerobic conditions without the *C. gigas* hemocytes affected the bacterial survival during the 60-min incubation period (data not shown).

Effect of DPI on intracellular killing of bacteria by the C. gigas hemocytes in an aerobic environment

When DPI was added to the aerobic hemocytes, their ability for intracellular killing was less efficient and was similar to that found in the anaerobic environment (Fig. 2). The addition of DPI partly but significantly diminished the intracellular killing of *A. ramosus* by the *C. gigas* hemocytes in the aerobic environment; the intracellular killing percents in the absence and presence of DPI were 64.8% and 37.4%, respectively (Fig. 2a). Under the anaerobic condition, however, the intracellular survival of *A. ramosus* was unaffected by the addition of DPI (Fig. 2b). On the other hand, for the killing of *M. luteus* by the aerobic *C. gigas* hemocytes, no significant difference was detected between the absence of DPI (48.9%) and the presence of DPI (50.7%) (Fig. 2a). In experiments under anaerobic conditions, it was found that DPI did not inhibit the killing of *M. luteus* by the *C. gigas* hemocytes (Fig. 2b). Therefore, these results indicate that the aerobic killing of *A. ramosus* by the *C. gigas* hemocytes is partly dependent on the NADPH oxidase activity of the hemocytes, whereas activation of the NADPH oxidase does not occur in the *M. luteus* killing process of the *C. gigas* hemocytes.

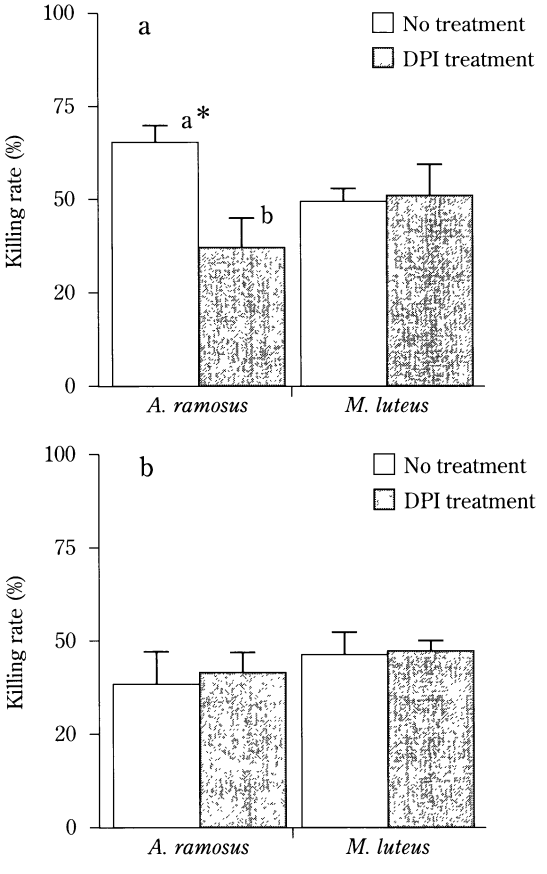

Fig. 2. Effects of DPI on intracellular killing activity of *C. gigas* hemocytes against *A. ramosus* and *M. luteus* in the aerobic (a) and anaerobic (b) environments. *The data expressed as columns having different alphabetical designations are significantly different using Fisher's LSD method ($p<0.05$; n=5).

The reduction in the intracellular killing of *A. ramosus* was not due to a decrease in the cell viability or phagocytic ability. There was no effect on trypan blue exclusion >90% after 60 min for both the untreated and DPI-treated hemocytes. Moreover, phagocytosis and the intracellular killing of *A. ramosus* were not significantly affected by the 0.1% DMSO, the solvent for DPI, when compared with the control containing oyster BSS.

Discussion

In mammalian leukocytes, much evidence has been accumulated to indicate that O_2^- and H_2O_2 play an important role in the oxygen-dependent bactericidal activity against ingested bacteria[16]. In mollusks, few studies concerning the intracellular killing of phagocytized bacteria by hemocytes are available, in particular in which oxygen-dependent killing mechanisms are included[4]. The present data demonstrated that the abilities of *C. gigas* hemocytes to phagocytize and to kill all tested bacterial species were clearly evident. However, the effects of the ROIs on the hemocyte-mediated bactericidal activity widely varied between that for *A. ramosus* and that of the other four bacterial species,

M. luteus, *D. pacifica*, *E. coli*, and *B. subtilis*.

Whereas viable *A. ramosus* strongly enhanced the hemocyte-generated PMAC CL, the four other bacterial species did not (Fig. 1). Since PMAC CL is considered to augment in response to the production of O_2^- and H_2O_2, *A. ramosus* could stimulate the ROI-generation system in the *C. gigas* hemocytes. The strain of *A. ramosus* used showed little or no catalase activity. It has been suggested that H_2O_2 is generated by the hemocytes in response to live *A. ramosus*, and that *A. ramosus* cannot scanvenge the produced H_2O_2. In contrast, because the other four bacteria did not elicit the ROI-production by hemocytes, we hypothesized that the ROI-dependent bactericidal capability toward these bacteria did not occur. However, we could not assess in this study whether the *C. gigas* hemocytes did not produce ROIs against the four tested bacterial strains or generated low levels of ROIs, which were overcome by the antioxidant systems in bacteria. These results are in agreement with the findings obtained by the serial investigations of Bramble and Anderson[7-9]. Lambert and Nicolas[17] reported that certain species of vibrios and *Alteromonas* enhanced the luminol-CL of *P. maximus* and *C. gigas* hemocytes, whereas these bacteria strongly inhibited the CL generated by hemocytes in response to zymosan. Zymosan is most potent stimulant to generate hemocyte luminol CL that is associated with ROI-generation[8]. Therefore, it is considered that these vibrios and *Alteromonas* strains are not necessarily inducing the phagocytic ROI-generation by the *P. maximus* and *C. gigas* hemocytes.

We showed that the intracellular killing of *A. ramosus* by the *C. gigas* hemocytes was markedly decreased from 66.2% to 36.7% under anaerobic conditions (Table 2). In contrast to *A. ramosus*, the four other species were attacked by the *C. gigas* hemocytes almost as efficiently under anaerobic as under aerobic conditions. Under the anaerobic conditions, the *C. gigas* hemocytes are incapable of generating reduced ROIs such as O_2^- and H_2O_2 and thus are usually incapable of killing bacteria via oxygen-dependent pathways. Therefore, it is considered that the percent reduction at which hemocytes killed *A. ramosus* under the anaerobic conditions correlates with no generation of the ROIs, especially H_2O_2, by the *C. gigas* hemocytes. This finding corresponded to the result that the intracellular killing of *A. ramosus* by the *C. gigas* hemocytes is repressed by the addition of DPI under aerobic conditions (Fig. 2). DPI is a potent inhibitor of NADPH oxidase[18], and it also inhibits the lucigenin-enhanced CL by *C. virginica* hemocytes[9]. We previously reported that the NADPH oxidase-like activity occurred in the *C. gigas* hemocytes, and its activity was completely inhibited when the *C. gigas* hemocytes were treated with a DPI concentration of $10\mu M$[11]. Accordingly, the repression of the killing ability in the *C. gigas* hemocytes by DPI treatment might be responsible for the non-generation of O_2^- resulting from the inactivation of the NADPH oxidase.

Elsbach and co-workers demonstrated that the susceptibility of bacteria to the cytotoxic effects of ROIs produced granulocytes widely varied between gram-negative and positive bacteria, even within species showing the same gram-stain characteristics[12,19]. The data obtained in this study indicated that different species of bacteria possessed different susceptibilities to the bactericidal effects of the *C. gigas* hemocytes. Furthermore, the results of this study support the work by other investigators[9,20]. It also extended our understanding and raised questions concerning the defense functions of the *C. gigas* hemocytes. Oxidative killing activity occurred in part due to the ROIs produced by the *C. gigas* hemocytes, however, its activity was shown to kill *A. ramosus* that was only one species among the five species of bacteria tested in this study. Apparently, our data reflected the existence of oxygen-independent bactericidal hemocyte functions. Furthermore, these data suggested that these functions mainly contribute to the intracellular killing activity of the hemocytes against the tested bacteria. Comparative studies of the oxidative and non-oxidative killing systems of the *C. gigas* hemocytes should further delineate the host defense mechanisms in *C. gigas*.

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マガキ血球の細胞内殺菌過程における活性酸素の関与

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マガキ血球が貪食した細菌に対する殺菌過程において，活性酸素の関与の有無を明らかにするため，好気・嫌気の両条件下において貪食能および殺菌能の測定を行った。供試した5種類の細菌株のうち，*Arthrobacter ramosus* のみが血球の活性酸素生成を有意に刺激した。血球の貪食能はいずれの細菌株に対しても好気・嫌気の2つの条件の間で違いはなかった。しかし，*A. ramosus* に対する血球の殺菌能は，嫌気条件下で約半分に低下した。殺菌能の同様の低下が，NADPH 酸化酵素の活性阻害剤 diphenyleneiodonium の添加によっても認められた。他の4種類の細菌株に対する殺菌能は，好気・嫌気の違いによって変化しなかった。従って，マガキ血球の *A. ramosus* に対する殺菌は部分的に活性酸素依存的であること，一方その他の細菌に対して血球の活性酸素生成系は反応せず，殺菌は酸素非依存的に起こることが考えられた。