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Review

Functional Profiles of Hemocytes in the bio-defense Process of the Pacific Oyster, *Crassostrea gigas*

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In mammals, phagocytic leukocytes (e.g., neutrophils, monocytes, and macrophages) contribute to the host-defense mechanism of the individual against invading microorganisms using a strong phagocytic activity. Unlike cytotoxic lymphocytes and the complement system, the phagocytic leukocytes non-specifically phagocytize and kill their target microorganisms. The phagocytic leukocytes generate reactive oxygen intermediates (ROIs) including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$), in response to phagocytic particles or soluble stimuli. These ROIs are reactive enough to destroy most biological molecules including invading microorganisms, therefore, they are considered to be an important bactericidal (microbicidal) process in the phagocytic leukocytes (reviewed by Babior, (1)). In fact, a correlation between the ROI production and microbicidal activity of the phagocytic leukocytes has been demonstrated in mammals (Sasada and Johnston, (2); Miyasaki *et al.*, (3); Hampton and Winterburn, (4)).

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 including potential pathogens and subsequently actively phagocytize these invaders (Alvarez *et al.*, (5); McCormick-Ray and Howard, (6); Lopez-Cortes *et al.*, (7)). The hemocytes of mollusks morphologically resemble mammalian phagocytic leukocytes (reviewed by Adema *et al.*, (8); Cheng, (9); (10)) and, like these leukocytes, have the ability to recognize, engulf, and internally degrade biological particles including foreign microorganisms (Foley and Cheng, (11)). Moreover, the generation of ROIs by hemocytes in response to zymosan particles and other nonliving solid stimuli have been demonstrated in several molluscan species (reviewed by Anderson, (12)). In the hemocytes of molluscs, however, some of the correlation details between the ROI generation and intracellular bactericidal ability have yet to be unequivocally demonstrated.

The objective of this review article is to present a synopsis about the occur-

rence of two subpopulations of hemocytes, phagocytosis, and the ROI-generating system of molluscan hemocytes, especially those of the Pacific oyster, *Crassostrea gigas*.

Oyster hemocytes: occurrence of two subpopulations and their phagocytic ability

Agranulocytes and granulocytes

The different froms and functions of bivalve molluscan hemocytes have been reviewed in depth by Cheng ((9); (10)). A classification of the hemocytes have resulted in the recognition of two categories of cells, which have been designated granulocytes and hyalinocytes (agranulocytes) (Cheng, (9)).

Granulocytes are distinguished from other hemocytes by the possession of many cytoplasmic granules. Also, many investigators reported that at least three subcategories of granulocytes occur in the hemolymph of several bivalve species including *C. gigas* (reviewed by Cheng, (10); Hine, (13)). The bases for this classification of subcategories are that granulocytes have three distinct types of cytoplasmic granules when observed under light and electron microscopes.

In order to recognize and to separate populations and/or subpopulations of the *C. gigas* hemocytes, the authors have being carried out several experiments: microscopic observations for live hemocytes with no stain under a phase contrast microscope and with supravital stain; light microscopic observations for hemocytes, which were spread on glass slides and fixed with different types of fixatives, with Giemsa, May-Grünwald, Wright, and hematoxylin stains; and continuous density gradient centrifugation with 60% Percoll. As a result of these microscopic studies, we recognized the following three types of agranular hemocytes and one type of granulocyte in *C. gigas*: common agranulocytes, fibrocytes (fibroblast-like cells), lymphoid agranulocytes, and granulocytes commonly including mixtures of acidophilic and basophilic granules. After a 60% Percoll gradient centrifugation, we could obtain a 95% pure fraction of granulocytes but could not separate the common agranulocytes, fibrocytes, and lymphoid agranulocytes. Therefore, we concluded that the hemocytes in *C. gigas* compose two subpopulations, namely, granulocytes and agranulocytes under our experimental conditions.

Cheng (10) described that the differences in ages, physiological states, and environmental factors influence the number of circulating hemocytes in each individual mollusks, cause large fluctuations in both the total number of hemocytes and the ratios between the agranulocytes and granulocytes. Therefore, the establishment of baseline counts of hemocytes in oysters or other molluscan species is difficult. To obtain data pertaining to the number and composition of the hemocytes, we investigated the annual changes in the

hemocytic density in hemolymph and the ratios between agranulocytes and granulocytes in *C. gigas* hanging-cultured in Onagawa Bay, Miyagi Prefecture, from August 1997 to July 1998 (Figs. 1 and 2). The hemocytic density exhibited a remarkable seasonal change (Fig. 1). The total hemocyte count in each *C. gigas* individual collected from the same hanging-place varied from 656 ± 170 (February) to $2{,}041 \pm 235/mm^3$ (June). We had previously obtained similar results when measuring the annual change in hemocytic density of *C. gigas* in Onagawa Bay from August 1994 to July 1995 (Takahashi, unpublished data) and from May 1996 to February 1997 (Ishikawa *et al.*, (14)). As described above, the number of circulating hemocytes is influenced by changes in the ambient environmental factors such as water temperature and salinity (Feng, (15); Fisher, (16)). As shown in Figure 1, in the groups collected from December to February, lower values of hemocytic density corresponded to the decline in the ambient temperature (6-9°C). However, in spite of the high temperature (23°C) in September, the hemocytic density significantly dropped. This decrease in hemocytic density is probably due to the influence of spawning. To understand the annual change in hemocytic density, in addition to the ambient environmental factors, consideration must be given to gonadal maturation and spawning.

The ratios between the agranulocytes and granulocytes also varied during the year, however, the number of agranulocytes was always greater than that of the

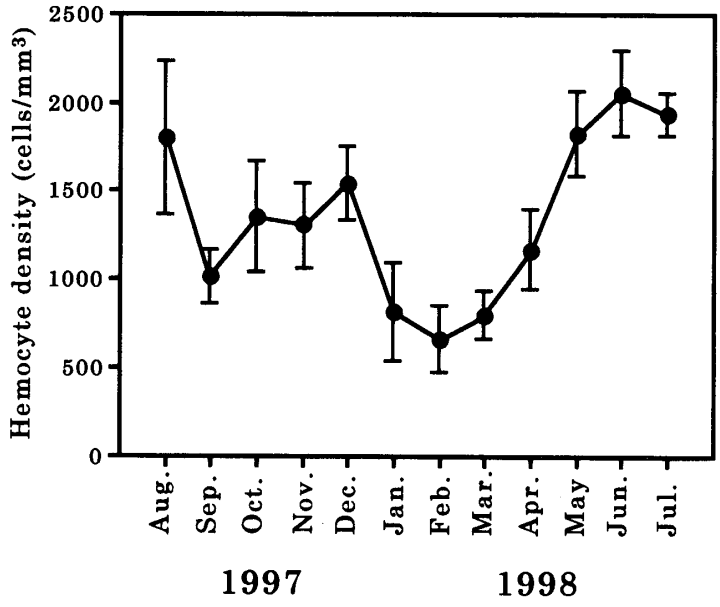

FIG. 1. Seasonal changes in density of hemocytes in the hemolymph of *Crassostrea gigas*. Samples were collected monthly from Onagawa Bay, Miyagi Prefecture, from August 1997 to July 1998. The number of hemocytes was counted on a Thoma hemocytometer under a light microscope. Each value is expressed as the mean number of hemocytes per mm^3 of the hemolymph ($n = 10$). Vertical bars are standard errors (SE) of the means.

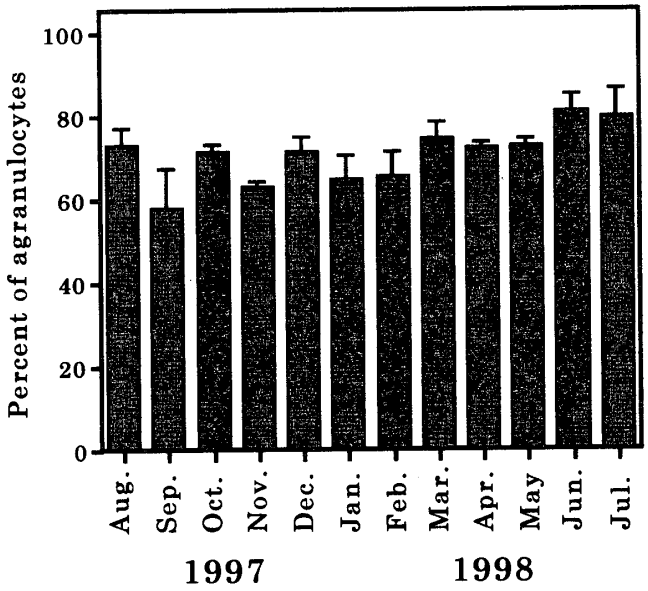

FIG. 2. Seasonal changes in the ratio of agranulocytes to total count of hemocytes in *C. gigas*. Samples were collected monthly from Onagawa Bay, Miyagi Prefecture, from August 1997 to July 1998. The agranulocyte population was discriminated using supravital staining with neutral red and janus green. Results are means±SE of ten individuals.

granulocytes (Fig. 2). The agranulocyte ratio varied from about 58.2% (September) to 80.5% (June) of the total number of hemocytes in *C. gigas* that were examined. On the other hand, in *C. virginica* hemocytes, the number of granulocytes is much greater than that of the agranulocytes. Foley and Cheng (11) reported that granulocytes comprised about 87.5% of the total number of hemocytes in *C. virginica*. Furthermore, McCormick-Ray and Howard (6) reported that the hemocytes in *C. virginica* were present at the granulocyte to hyalinocyte (agranulocyte) ratio of 61% : 39%. With the exception of the difference in species, the apparent discrepancy between *C. gigas* and *C. virginica* is due to the difference in the classification of the hemocytes. Foley and Cheng (11) categorized the granulocytes as typical granulocytes plus fibrocytes, while we classified the fibrocytes into the agranulocyte subpopulation.

Phagocytosis

The phagocytic process of the hemocytes is characterized by the following four phases (Ceng, (10); Torreilles *et al.*, (17)): (1) recognition of non-self materials, (2) binding of non-self materials to hemocytes (surface attachment), (3) engulfment of non-self materials into phagosomes, and (4) intracellular killing and degradation of non-self materials in most instances. The capability of the hemocytes of *C. virginica* and the mussel *Mytilus edulis* to phagocytize and intracellularly degrade bacteria has been well reviewed by Feng (18). Feng's

review article suggested that the presence of host defense mechanisms was mediated by phagocytosis against invading bacteria. In the 4th phase of the phagocytic process, non-self materials within the phagosomes may be subjected to hydrolytic lysosomal enzymes and ROIs, which serve a role in destroying the non-self materials (Adema *et al.*, (19); Anderson *et al.*, (20)).

Hine (13) summarized the phagocytic characterization by both agranulocytes and granulocytes: granulocytes exhibit a high phagocytic ability against various foreign particles (Pipe, (21); Anderson, (22)); on the other hand, agranulocytes may have a non-phagocytic ability in *M. galloprovincialis* (Carballal *et al.*, (23)) and *Tridacna crocea* (Nakayama *et al.*, (24)), a lower phagocytic ability than granulocytes (Renwrantz *et al.*, (25); Tripp, (26)). We also examined the phagocytic ability of both agranulocytes and granulocytes against three species of bacteria, formalinized erythrocytes, heat-killed yeast cells, and latex beads. Both the agranulocytes and granulocytes exerted phagocytic ability against all the bacterial strains tested (Fig. 3). Granulocytes were more active phagocytes against all species of bacteria tested and the phagocytic rate renged from 12.9% to 44.6% of the agranulocytes and from 48.0% to 64.6% of the granulocytes. Yeast cells were also extensively phagocytized by granulocytes, but the agranulocytes produced a little phagocytosis of yeast cells (Table 1). On the other hand, agranulocytes were more phagocytically active than granulocytes against erythrocytes and latex beads (Table 1). These results suggest that most foreign particles, if not all, are more actively phagocytized by granulocytes than by agranulocytes. Moreover, we examined the effects of various treatments for

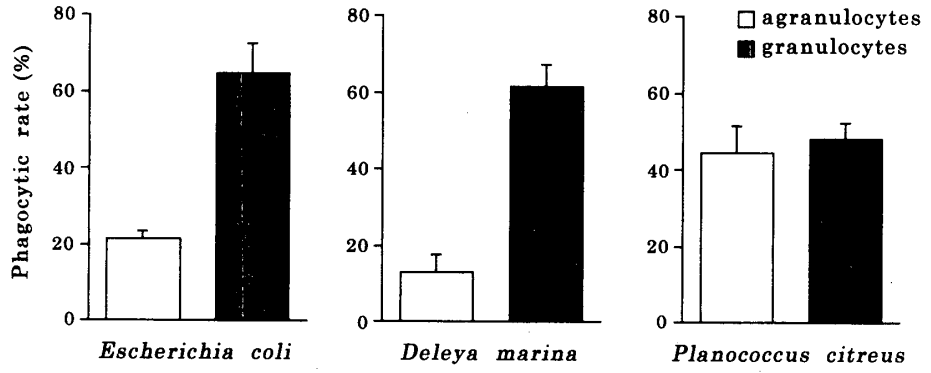

FIG. 3. Phagocytosis of three species of bacteria by *C. gigas* hemocytes. The extent of phagocytosis was determined by randomly counting at least 400 hemocytes from each monolayer under an oil immersion lens of a light microscope. Three species of bacteria, *Escherichia coli*, *Deleya marina*, and *Planococcus citreus*, were tested as foreign particles. The percent exhibiting phagocytosis (phagocytic rate) was calculated as

$$\frac{\text{number of hemocytes engulfing at least one bacterium}}{\text{total number of hemocytes counted}} \times 100$$

Results are means $\pm$ SE of five separate experiments performed in triplicate.

TABLE 1. *Phagocytosis of various particles by C. gigas hemocytes*

Particle	Agranulocyte		Granulocyte	
	PR (%)	PI	PR (%)	PI
Human erythrocytes	34.3	3.21	22.4	1.57
Yeast cells	26.1	2.14	75.1	7.72
Latex beads ($\phi 2\ \mu$m)	58.5	11.08	40.6	3.95

Hemocyte monolayers and foreign particles were co-incubated for 60 min at 20°C.
The percent exhibiting phagocytosis (phagocytic rate, PR) was same as that in Fig. 3.
Phagocytic index (PI) was expressed as the average number of particles ingested in one hemocyte.
Results are means of six separate experiments performed in triplicate.

the same foreign particles on the phagocytic ability of both the agranulocytes and granulocytes (Fig. 4). Yeast cells were avidly phagocytized by granulocytes whether fresh or fixed yeast cells were used. Also, there was no appreciable effect on the phagocytosis of yeast cells by granulocytes, if the yeast cells were killed by heating or autoclaving. In contrast, *Escherichia coli* cells treated with formaldehyde and then autoclaved were strongly resistant to phagocytosis by granulocytes while living cells or heat-killed cells were avidly phagocytized. On the other hand, the phagocytosis of both yeast and *E. coli* cells by agranulocytes was not affected by the treatments used in this study. These results suggest that a chemical or physical alteration of the cell surfaces of *E. coli* affects their ability to be engulfed by granulocytes, but not by the agranulocytes. Tripp (26) reported similar results in relation to phagocytosis by *Mercenaria mercenaria* hemocytes. It is also suggested that granulocytes may possess a specialized system mediated by a membrane receptor in a non-self recognition toward foreign particles, similar

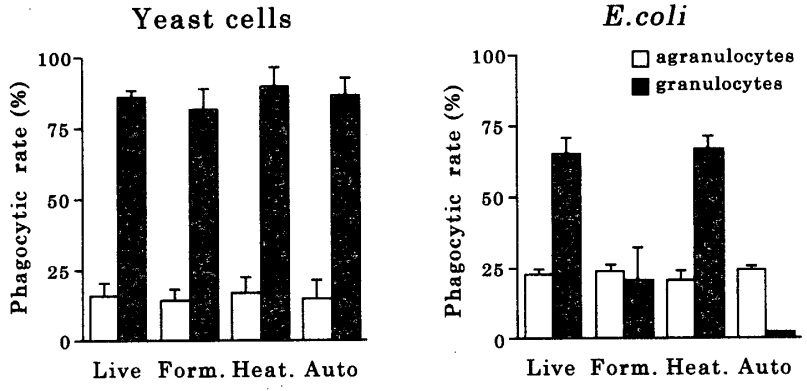

FIG. 4. Phagocytosis of yeast cells and *E. coli* after no treatment (Live), formalinization (Form.), heating at 100°C for 60 min (Heat.), and autoclaving at 121°C for 20 min (Auto.). The measurement methods of phagocytosis by *C. gigas* hemocytes were same as those in Fig. 3.

to that of the professional phagocytes in mammals.

ROI-generating systems in the hemocytes of mollusks

Phagocytosis by mammalian macrophages and neutrophils activate a membrane-bound oxidase, and is accompanied by increase in oxygen uptake (the 'respiratory burst'). In this respiratory burst process, an initial biochemical event during the ROI production is the conversion of molecular oxygen (O_2) to O_2^- catalyzed by the NADPH oxidase system (Makino *et al.*, (27)). The NADPH oxidase system in mammalian phagocytic leukocytes is well known to be composed of serial redox components (reviewed by Segal and Abo, (28)).

Recent investigations have demonstrated the generation of ROIs by stimulated hemocytes in several molluscan species: O_2^- and/or H_2O_2 generation by hemocytes from *M. edulis* (Pipe, (21); Noël *et al.*, (29)); *C. virginica* (Larson *et al.*, (30); Anderson *et al.*, (31), (32); Friedl and Alvarez, (33); Austin and Paynter, (34)); *C. gigas* (Bachère *et al.*, (35); Takahashi *et al.*, (36)); the European flat oyster, *Ostrea edulis* (Bachère *et al.*, (35)); the scallop, *Patinopecten yessoensis* (Nakamura *et al.*, (37)); and the gastropod mollusks, *Achatina achatina*, *A. fulica*, *Helix aspersa*, and *Lymnaea stagnalis* (Dikkeboom *et al.*, (38), (39); Adema *et al.*, (19)). The oxidative activity in molluscan hemocytes has been shown to be valuable in defining host-defensive events (Adema *et al.*, (8); Anderson, (22); Greger *et al.*, (40)) and hemocyte-pathogen interactive modulation (Hervio *et al.*, (41); Le Gall *et al.*, (42); Bramble and Anderson, (43), (44)).

The ROI production that probably depends on NADPH oxidase (NADPH oxidase-like enzyme) has been confirmed in the hemocytes of *L. stagnalis* (Adema *et al.*, (45)), *C. virginica* (Bramble and Anderson, (46)), and *C. gigas* (Takahashi and Mori, (47)) as well as in mammalian phagocytic leukocytes. A rapid increase in O_2 consumption was observed accompanying a cyanide-independent respiratory burst when *C. gigas* hemocytes were stimulated with phorbol myristate acetate (PMA) (Takahashi and Mori, (47)). The chemiluminescent response depending on the ROI generation by zymosan- or PMA-stimulated hemocytes of these three species was strongly inhibited by the addition of diphenyleneiodonium, a specific inhibitor of mammalian NADPH oxidase. Moreover, we demonstrated using the electron spin resonance (ESR) spin trapping and the chemiluminescence (CL) method that *C. gigas* hemocytes stimulated with PMA exclusively generated O_2^- as the primary metabolite of O_2 (Takahashi *et al.*, (36)).

Therefore, as described above, the following hypothesis has been generated: these phagocytic hemocytes of mollusks possess a specific ROI-forming system associated with NADPH oxidase, which becomes functional due to the stimulation mediated by the phagocytosis of foreign particles and microbes, and the ROIs contribute to the bactericidal action of hemocytes, which is similar to the professional phagocytic leukocytes in mammals (Adema *et al.*, (8); Anderson *et al.*,

(20)). However, in serial investigations performed by Bramble and Anderson ((43) ; (44)), *C. virginica* hemocytes stimulated by the bacterium *Listonella anguillarum* produced low levels of ROIs, which may by incapable of surpassing the bacterial antioxidant capability. Moreover, Bramble and Anderson (46) demonstrated that *C. virginica* hemocytes did not generate ROIs upon stimulation with the bacteria *Bacillus megaterium* and *Pseudomonas fluorescence*, whereas the bactericidal activity of *C. virginica* hemocytes against these bacteria was clearly evident. These results suggested that ROIs generated by *C. virginica* hemocytes do not participate in tha killing of these three species of bacteria as bactericidal effectors in the host-defense system of oyster hemocytes.

Therefore, we have being carried out several studies aimed at understanding the systems in the ROI-generating of *C. gigas* hemocytes (Takahashi *et al.*, (36) ; Takahashi and Mori, (47) ; Takahashi, unpublished data). As a result, differences in the potential for O_2^- generation occurred in an intersubpopulation (between agranulocytes and granulocytes) way. Granulocytes produced large amount of O_2^- after PMA stimulation, but agranulocytes did not or only slightly produced O_2^- after PMA treatment (Table 2). Noël *et al.* (29) also reported that the zymosan-stimulated luminol CL was the most intense of the eosinophilic granulocytes compared to the basophilic granulocytes and hyalinocytes in *M. edulis*.

Both the agranulocytes and granulocytes showed H_2O_2 generation associated with the phagocytosis of zymosan (Table 2). Resting *C. gigas* hemocytes exhibited a low level of H_2O_2 generation ; the H_2O_2 level was significantly increased by the addition of zymosan. Similar results were reported in the hemocytes of *L. stagnalis* (Adema *et al.*, (19)) and *M. edulis* (Pipe, (21)). We also confirmed in

TABLE 2. *O_2-and H_2O_2 generation phagocytosis-associated by C. gigas hemocytes*

O_2-(Cytochrome *c* reduction)

	Cytochrome *c* reduction (nmol/10^6 hemocytes/90 min)		
	Resting	Phagocytizing	Phagocytizing+SOD§
Agranulocyte	1.3±0.11	1.9±0.68	1.6±0.43
Granulocyte	5.3±0.91	17.6±2.94*	8.9±1.21

H_2O_2 (Scopoletin oxidation)

	Scopoletin oxidation (nmol/10^6 hemocytes/90 min)	
	Resting	Phagocytizing
Agranulocyte	24.4±6.91	47.8±9.46*
Granulocyte	19.3±5.27	70.6±9.15*

* Significantly different from resting ($p<0.01$).

§ SOD ; superoxide dismutase

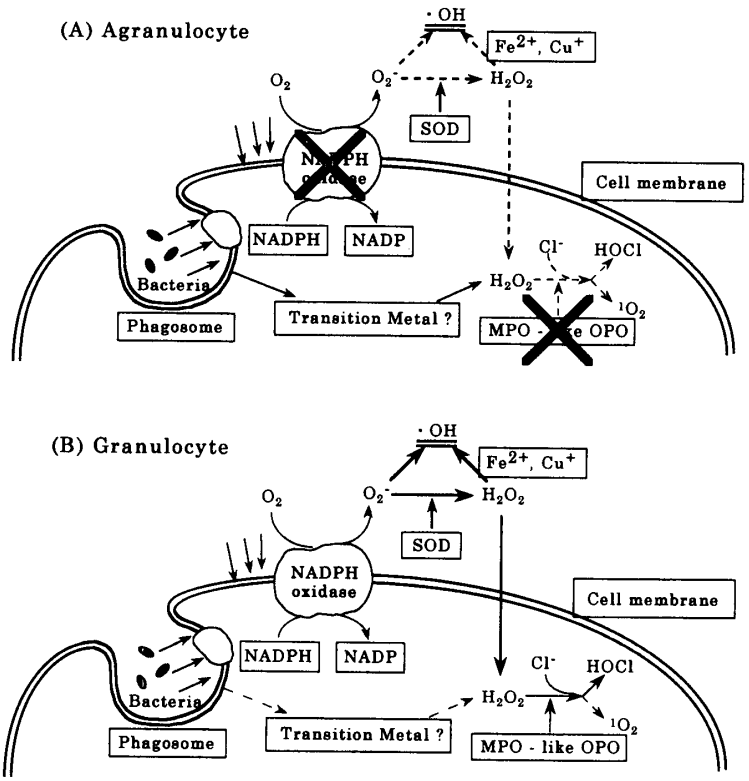

FIG. 5. Postulated pathway for generation of reactive oxygen intermediates by *C. gigas agranulocytes* (A) and granulocytes (B).

granulocytes that •OH could be produced by the reaction of O_2^- and H_2O_2 (Takahashi and Mori, unpublished data). Granulocytes is rich in transition metals, such as Fe^{2+} and Cu^+, and these metals may act as reducing agents in the formation of •OH. H_2O_2 plays an important role as a substrate for myeloperoxidase (MPO) for the production of hypochlorous acid (HOCL) which is well known to be a powerful oxidant and microbicidal agent. We found MPO-like activity (OPO activity) in *C. gigas* granulocytes, but not in agranulocytes. The MPO activity has been measured in *M. edulis* hemocytes (Schlenk *et al.*, (48); Coles and Pipe, (49)), and may also be found in *C. virginica* hemocytes (Anderson, (12)). Presumably, the MPO/H_2O_2/chloride system may be present in *C. gigas* granulocytes and in other bivalve hemocytes. Figure 5 shows that postulated pathway for ROI generation by both the agranulocytes and granulocytes.

In summary, hemocytes in *C. gigas* and other bivalves have a strong phagocytic ability against various foreign particles and microbes and a potent microbicidal action. Moreover, hemocytes possess specific ROI-generating systems, which are similar to those of professional phogocytes in mammals. However, in bivalve molluscan hemocytes, the precise roles and functional significance of ROIs in host-defense responses are still not completely understood.

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