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Characterization of the Chemiluminescence Measured in Hemocytes of the Eastern Oyster, *Crassostrea virginica*

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ABSTRACT Hemocytes of the Eastern oysters, *Crassostrea virginica*, produce reactive oxygen intermediates (ROIs) during phagocytosis to destroy foreign cells. Although evidence suggests that oyster hemocytes generate superoxide anions (O_2^-) following phagocytic stimulation, the production of other ROI species such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl radical (•OH), and hypochlorous acid (HOCl) has not been directly investigated. In this study, ROI production by oyster hemocytes was measured by quantifying the production of chemiluminescence (CL) amplified with different luminescent probes. The ROIs involved in CL production were identified by testing the effects of scavengers and inhibitors of particular ROI species on CL. Taurine completely inhibited luminol-dependent CL, indicating that HOCl production was primarily responsible for the CL generated by hemocytes during phagocytosis of yeast granules. In addition, azide strongly inhibited luminol CL, implicating the involvement of myeloperoxidase in the production of HOCl and the resulting CL. Superoxide dismutase partially inhibited CL, indicating that superoxide ions were also produced and contributed to CL. Luminol CL was significantly higher in hemocytes from oysters heavily infected with the parasite *Perkinsus marinus* and remained completely inhibitable by taurine suggesting that HOCl production was enhanced by infection. Last, the concentration of taurine measured in oyster hemolymph was sufficient to quench a significant amount of the HOCl generated by the hemocytes and may reduce the effectiveness of the oyster's defense response to infections.

The disease defense system of oysters consists of both cellular and humoral responses (Feng, '88; Adema et al., '91a). Phagocytic cells called hemocytes are the primary cells involved in defense and are responsible for the destruction of foreign cells. Phagocytic stimulus triggers a biochemical cascade resulting in the release of lysosomal enzymes and reactive oxygen intermediates (ROIs), both of which destroy invading cells (Cheng, '83, '84; Adema et al., '91a; Anderson et al., '92a). Vertebrate phagocytes such as monocytes, macrophages, and polymorphonuclear leukocytes (PMNs) produce a variety of ROI species during phagocytosis including superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl radicals (•OH) and hypochlorous acid (HOCl) (Stevens et al., '78; DeChatelet et al., '82; Seim, '83; Allen, '86; Gyllenhammar, '89). These ROIs inflict a variety of damaging biochemical effects on foreign cells including lipid peroxidation, destruction of membrane integrity leading to cell lysis, protein degradation and enzyme inactivation, oxidation of amino acids and reducing agents, DNA strand breakage, and weak mutagenesis (Thomas et al., '88; Afanas'ev, '91; Anderson et al., '92a). The extensive cellular damage caused by these oxidants makes them potent killing agents of harmful microorganisms such as viruses, bacteria, and protozoa (Klebanoff, '68; Washburn et al., '87; Thomas et al., '88; Marodi et al., '91). Therefore, the production of ROIs by oyster hemocytes may play an important role in the oyster's defense response to infections.

The production of ROIs in vertebrate phagocytes is initiated by the activation of oxidant-generating enzymes such as NADPH oxidase, superoxide dismutase (SOD), and myeloperoxidase (MPO). NADPH oxidase, a membrane-bound flavoprotein oxidase, generates superoxide anions by the one-electron reduction of oxygen at the expense of NADPH (Heyworth et al., '89; Ohtsuka et al., '90). Superoxide anions are subsequently converted to H_2O_2 by SOD (Afanas'ev, '91). Myeloperoxidase, an intracellular enzyme contained within primary

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lysosomes, catalyses the production of HOCl and water from H_2O_2 and Cl^- (Klebanoff, '68; Ohno and Gallin, '85; Andrews and Krinsky, '85). All of the ROIs produced in this series of reactions are potent oxidants.

Chemiluminescence (CL) measured with luminescent probes such as luminol is commonly used to measure the production of ROIs by molluscan hemocytes (Adema et al., '91a,b). For instance, hemocytes from oysters infected with the parasitic protozoan, *Perkinsus marinus*, produce higher levels of luminol-dependent CL during phagocytosis of zymosan granules, indicating that ROI production is enhanced as a result of infection (Anderson et al., '92a). Hemocytes of both *Crassostrea virginica* and *Crassostrea gigas* produce superoxide anions following phagocytic stimulation with yeast granules or chemical stimulation with phorbol 12-myristate 13-acetate (PMA) (Chagot, '89; Takahashi et al., '93; Anderson et al., '92b; Anderson, '94). In addition, hemocytes of gastropod molluscs (*Lymnaea stagnalis*, *Helix aspersa*, and *Biomphalaria glabrata*) display superoxide production during phagocytosis (Shozawa, '86; Dikkeboom et al., '87, '88). Furthermore, NADPH oxidase activity has been demonstrated on the plasma membrane of *Ostrea edulis*, *C. gigas*, and *L. stagnalis* hemocytes (Chagot, '89; Adema et al., '93). Although this evidence suggests that oyster hemocytes produce superoxide anions during phagocytosis, the production of other ROI species such as H_2O_2, $^1O_2$, $\cdot OH$ and HOCl has not been investigated.

The reports cited above suggest that the production of superoxides might account for the CL generated by oyster hemocytes during phagocytosis. Luminol, however, is a non-specific probe capable of reacting with a number of different ROI species (Allen, '86; Gyllenhammar, '89; Afanas'ev, '91). In vertebrate phagocytes, luminol-dependent CL produced following phagocytic stimulation is caused by increased H_2O_2 production and the subsequent generation of HOCl by MPO (Stevens et al., '78; DeChatelet et al., '82; Seim, '83; Allen, '86). While the production of HOCl by molluscan hemocytes has not previously been tested, putative MPO activity was demonstrated in hemocytes from the bivalve *Mytilus edulis* (Schlenk et al., '91). In addition, hemocytes of other molluscs (*Pactinopecten yessoensis* and *L. stagnalis*) generate hydrogen peroxide, a substrate for MPO (Nakamura et al., '85; Dikkeboom et al., '87, '88). Therefore, the CL produced by oyster hemocytes during phagocytosis may also represent HOCl production.

The purpose of this study was to characterize the production of CL by *C. virginica* hemocytes during phagocytosis. The ROIs involved in CL production were identified by testing the effects of scavengers and inhibitors of particular ROI species on CL. The results of these measurements indicated that the production of HOCl was primarily responsible for luminol CL generated by oyster hemocytes during phagocytosis of zymosan granules. In addition, luminol CL was significantly higher in hemocytes from oysters heavily infected with *P. marinus*, suggesting that infection induced increased HOCl production. It is important to note that HOCl will readily react with taurine to form N-chlorotaurine which, unlike other chlorinated amino acid compounds, is very stable (Zgliczynski et al., '71; Brestel, '85; Reddy et al., '89). Since taurine is normally present in high concentrations in oyster tissues, especially at high salinity (Lynch and Wood, '66; Pierce et al., '92; Paynter et al., '95), the concentration of taurine in the hemocytes, hemolymph, and gill tissue was quantified to evaluate its possible effects on ROI production within the oyster. The amount of taurine measured in the hemolymph was sufficient to quench substantial amounts of the HOCl produced by the hemocytes and, thus, may reduce the effectiveness of this defense mechanism.

MATERIALS AND METHODS

Animals

Crassostrea virginica were collected from various locations of differing salinities and *Perkinsus marinus* prevalences within the Chesapeake Bay. Typically, oysters from low salinity had light infections, while those from higher salinities had moderate-to-heavy infections. Oysters were maintained in aerated aquaria containing artificial seawater (Instant Ocean) at 25°C and the salinity of the collecting site (20–35 ppt). Oysters were not fed and kept in the aquaria for at least 5 days before hemocytes were harvested.

In some experiments, oysters from the Thames River, Connecticut, were used as noninfected controls when it was impossible to find noninfected Chesapeake Bay oysters, especially from August to November when disease prevalence in the Bay was at its highest. None of the Thames River oysters were infected with *P. marinus*.

Isolation of hemocytes

Oyster hemocytes were isolated by notching the valves, draining the mantle fluid, and withdraw-

ing hemolymph from the adductor muscle sinus with a syringe. Oysters were then set aside for *P. marinus* diagnosis. The hemolymph from a number of oysters was pooled and placed in test tubes on ice to prevent cell clumping. After all the hemolymph was collected, it was transferred into plastic petri dishes (10 ml/dish) and incubated at room temperature for 15 min, during which time the hemocytes adhered to the bottom of the dishes. The serum was then poured off, and the adherent cells were gently washed with filtered (0.2 μm) artificial seawater (FASW) prepared from Instant Ocean at ambient salinity. The cells were incubated in FASW for 2.5 h and the washed off the dishes by vigorous pipetting. The cells were resuspended in FASW, centrifuged (200–225*g*, 20°C) for 20 min, and resuspended in a small volume of a cell support media (CSM) consisting of 0.5% antibiotic/antimycotic solution (10,000 U/ml penicillin, 10 mg/ml streptomycin, and 25 μg/ml amphotericin B), 5% fetal bovine serum, and 1 mg/ml glucose in FASW (Anderson et al., '92a). all chemicals were obtained from Sigma (St. Louis, MO).

A hemocytometer was used to determine the number of cells per milliliter of suspension. The suspension was adjusted to 1×10^6 cells/ml with CSM. In experiments on hemocytes from individual oysters where hemolymph was not pooled, the final hemocyte pellet for each oyster was resuspended in 1 ml CSM, and the cell density was determined.

Measurements of chemiluminescence (CL)

The production of ROIs by oyster hemocytes was measured by quantifying CL augmented with the luminescent probes, luminol (5-amino-2,3-dihydro-1,4-phthal-azinedione) or lucigenin (bis-N-methylacridinium nitrate). Luminol augments CL from a variety of ROI species, whereas lucigenin is reported to be selective for detecting superoxide radicals (Stevens et al., '78; Allen, '86; Gyllenhammar, '89; Afanas'ev, '91). Hemocyte suspensions containing approximately 1×10^6 cells were added to 20-ml glass scintillation vials with either 10 μM luminol or 10 μM lucigenin. Luminol was prepared in a solution of 7.8% KOH and 6.2% boric acid in distilled water, while lucigenin was dissolved in distilled water (Scott and Klesius, '81; Allen, '86). Both probes were initially prepared at a concentration of 7.9 mM and stored in 1-ml aliquots at –70°C for no more than 2 weeks. Prior to assays, aliquots were thawed, and the probes were diluted to 1 mM with distilled water and kept in the dark. Control cell suspensions without the probes were also prepared to assess non-augmented CL. In addition, vials containing the probes in CSM without cells were prepared to determine if any excitation of the probes occurred in the absence of the hemocytes.

Chemiluminescence was measured with a scintillation counter (LKB Wallac 1219 Rackbeta) programmed for single photon monitoring (out of coincidence mode). Vials were counted repetitively in the dark at 1-min intervals for 20–30 min to establish background CL. Hemocytes were then stimulated by the addition of either zymosan (1 mg/ml) or phorbol 12-myristate 13-acetate (PMA) (10 μg/ml) to the vials, and counting was immediately resumed at 1-min intervals for 2 h. Zymosan, a preparation of *Saccharomyces cerevisiae* cell wall (Sigma), was used as a phagocytic stimulus, and PMA was used to chemically stimulate the production of ROIs by hemocytes.

Zymosan was prepared by boiling (1 g/100 ml FASW) for 30 min, centrifuging (480*g*, 20°C) for 10 min, and washing with FASW. The solution was centrifuged and washed two more times, then centrifuged again at the same speed and resuspended in FASW at 50 mg/ml. The washed zymosan was stored in 1-ml aliquots at –70°C until use, when it was thawed, centrifuged again, and resuspended in fresh CSM at 50 mg/ml (Anderson, '94). PMA was dissolved in 95% ETOH at 10 mg/ml and stored at –20°C, then diluted with CSM to 1 mg/ml. Either CSM or dilute ETOH in CSM were added to other hemocyte suspensions to ensure that stimulation by zymosan or PMA was responsible for changes in CL.

Chemiluminescence was quantified by plotting the curve of the counts per minute (cpm) (background corrected) following stimulant addition and then calculating the area under the curve. Because the CL responses of the various treatments were inherently different in both time and amplitude, the areas were normalized and expressed as cpm/min/cell to make direct comparisons between the treatments. Significant differences between the various CL responses with regard to probe or stimulant were assessed by two-factor analysis of variance (ANOVA) with significance at $P \leq .05$ (SuperANOVA; Abacus Concepts, Inc.; Berkeley, CA). Since the CL data were not normally distributed, ANOVA was conducted on transformed data ($\ln(1 + x)$).

To determine the relationship between ROI production and infection intensity, CL was quantified as described above for hemocytes isolated from infected and noninfected oysters. In experiments where CL was measured for hemocytes

from individual animals, infection intensity was determined for each animal. In experiments in which hemocytes were pooled from a number of oysters, the average intensity of infection within the sample of oysters used was calculated. Differences in the CL responses associated with infection intensity were assessed by a three-factor ANOVA among probe, stimulant, and infection intensity on the log transformed data.

Scavenger and inhibitor studies

Several ROI scavengers (superoxide dismutase, catalase, mannitol, sodium azide, and taurine) were used to determine which ROI species were responsible for the CL produced by oyster hemocytes (Table 1). The concentrations of the scavengers and inhibitors used were those previously shown to be effective in CL experiments on mammalian phagocytes and/or molluscan hemocytes.

Cell suspensions were preincubated with the scavengers in scintillation vials in the dark for 30 min prior to the addition of luminol or lucigenin. The viability of the hemocytes in the presence of the scavengers was tested by dye exclusion experiments using Trypan blue solution (0.4%). Cell viability was between 90 and 95% in all experiments. The probes were then added, and CL was measured before and after addition of either zymosan or PMA as described above. Control cell suspensions were also prepared for each experiment to which the scavengers were not added. In addition, vials were prepared containing CL probes and scavengers without cells. Last, the production of CL by cells incubated with the scavengers in the absence of probes was tested.

The control CL responses produced by hemocytes without the scavengers were normalized by setting them to 100%. The effects of the scavengers were expressed as a percentage of the normalized CL response (± SEM). Statistical significance was determined by using a single-sample t-test with the population mean (μ) equal to 100 (StatView II; Abacus Concepts, Inc.; Berkeley, CA). $P \leq .05$ was considered significant. In addition, a two-factor ANOVA was performed to determine if the effects of the treatments on CL were dependent on infection intensity.

TABLE 1. ROI scavengers and inhibitors employed in this study

Scavengers	ROI species	Concentration	Reference[1]
SOD	O_2^-	600–2,400 U/ml	4,1,2,11,8
Catalase	H_2O_2	500 U/ml	5,9,12,6,2
Mannitol	OH	1 mM	3,7
Azide	$^1O_2$/MPO	10 mM; 100 mM	3,7
Taurine	HOCl	0.025–10 mM	1,7,10

[1] 1) Albrecht and Jungi ('93); 2) Allen ('86)); 3) Brestel ('85); 4) Broberg and Pick ('85); 5) DeChatelet et al. ('82); 6) Dikkeboom et al. ('88); 7) Gyllenhammer ('89); 8) Ischiropoulos et al. ('89); 9) Sagone et al. ('77); 10) Stevens et al. ('78); 11) Williams and Cole ('81); 12) Takahashi et al. ('93).

Quantification of taurine

Taurine concentrations were measured in hemocytes, hemolymph, and gill tissue. Samples were prepared from Chesapeake Bay oysters kept at a salinity of 25 ppt. Hemolymph was withdrawn from several oysters and pooled. Hemocytes were isolated from the hemolymph as described previously, collected in FASW, and centrifuged (200–225*g*) for 20 min to obtain a hemocyte pellet. Gill tissue was removed from individual oysters, freeze-dried, and weighed. The hemocyte pellet and dried gill tissue were suspended in 40% ETOH and homogenized with a motor-driven teflon homogenizer (Tri-R, S63C; speed 6), boiled for 10 min to precipitate protein, and centrifuged (20,000*g*) for 15 min. The hemolymph was diluted in half with 80% ETOH, boiled, and centrifuged. All supernatants were freeze dried and the residue dissolved in lithium citrate buffer (pH 2.2). Taurine concentrations were determined with an automatic amino acid analyzer (Beckman, System Gold) (Pierce et al., '92).

Perkinsus marinus *infection diagnosis*

Perkinsus marinus infection intensities were determined for all oysters by the thioglycollate method (Ray, '52, '66). After oysters were bled, the valves were opened, and the rectum was carefully dissected out. The tissue was placed in a microfuge tube containing 80% thioglycollate medium (Difco) and 20% penicillin/streptomycin solution (Sigma) and incubated in the dark at 27°C for 7–10 days to allow for the development of hypnospores (Burreson, '91). Following incubation, tissue was removed, placed on glass microscope slides, and macerated with a scalpel. The tissue was then stained with filtered Lugol's iodine solution (50% KI and 40% I in distilled water) and examined under a light microscope for the presence of *P. marinus* cells which stain purplish-black. Infection intensity was rated on the basis of the number of parasite cells in several microscopic fields as light, moderate, or heavy; less than 50 cells per field was judged as light, between 50 and 100 cells as moderate, and 100 to 200 cells as moder-

ate/heavy. Tissues from some individuals were found to have many hundreds of cells per field, and these were scored as very heavy. Infection intensity was assigned the following number rating: none = 0, light = 1, moderate = 3, moderate/heavy = 4, and very heavy = 5.

RESULTS

Description of typical CL responses

Chemiluminescence produced by oyster hemocytes differed significantly depending on the type of probe and stimulant employed (two-factor ANOVA; $P < .05$; Figs. 1, 2). The background CL generated by hemocytes with luminol was one to two orders of magnitude higher than that with lucigenin. The addition of zymosan to hemocytes induced a rapid increase in luminol CL, reaching a peak within the first 10 min, approximately 10 times higher than the background CL (Fig. 1). Following the peak, CL gradually declined over a period of 120 min approaching background. When hemocytes were stimulated with PMA, luminol CL increased more slowly, reaching a maximum CL 20–60 min following stimulation, which was maintained for up to 60 min before declining (Fig. 1).

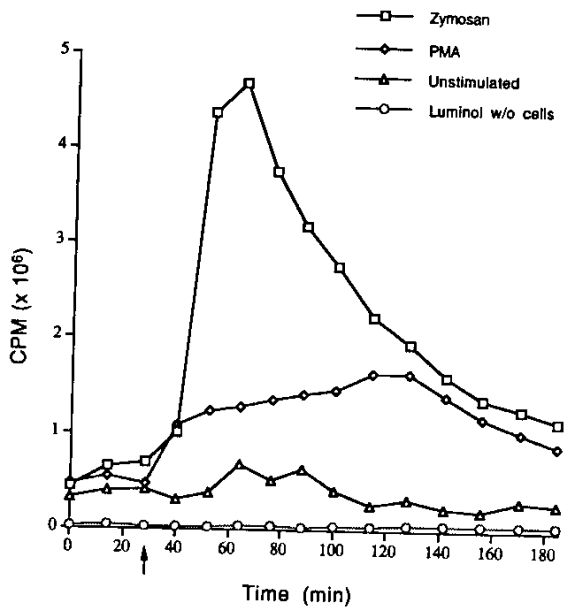

Fig. 1. Luminol-dependent chemiluminescence produced by oyster hemocytes at rest and following stimulation with either zymosan or PMA indicated by the arrow. The maximum luminol CL stimulated by zymosan was 5 to 10 times higher compared to that stimulated by PMA (two-factor ANOVA; $P = .01$).

The maximum luminol CL stimulated by zymosan was 5 to 10 times higher compared to that stimulated by PMA ($P = .01$; two-factor ANOVA among probe and stimulant).

Lucigenin CL stimulated by zymosan or PMA was significantly lower than the CL detected with luminol ($P = .0003$; two-factor ANOVA among probe and stimulant) (Fig. 2). In addition, PMA stimulated resulted in a gradual increase in lucigenin CL with a plateau 60–120 min after addition. This plateau was maintained for up to 100 min before a decline occurred. When hemocytes were stimulated with zymosan, a similar increase in lucigenin CL occurred with a plateau 30–60 min following addition. There was no difference in the magnitude of the total lucigenin CL produced following stimulation with either PMA or zymosan (Fig. 2). In both cases, maximum CL was approximately 50% higher than the background; however, the increases in CL detected with lucigenin were much lower than those detected with luminol.

No measurable CL was detected in cell suspensions without the probes. While the probes without cells generated some CL, the signal was lower than the background CL with cells and gradually declined over time. Unstimulated cells maintained a constant level of background CL in the presence of either probe, confirming that the increases in CL were the result of stimulation by zymosan or PMA (Figs. 1, 2).

Inhibition of CL by ROI scavengers

Superoxide dismutase, a scavenger of superoxide anions, caused a relatively low but significant inhibition of luminol CL stimulated by both zymosan (5% at 1,200 U/ml and 36% at 2,400 U/ml) and PMA (14% and 23%, respectively) (Table 2). Lucigenin CL stimulated by PMA was also inhibited by SOD at concentrations from 600 to 2,400 U/ml. However, lucigenin CL stimulated by zymosan was only significantly inhibited by SOD at 2,400 U/ml. Azide, an inhibitor of MPO and a scavenger of singlet oxygen, strongly inhibited luminol CL stimulated by both zymosan (40% at 10 μM and 80% a 100 μM) and PMA (60% and 66%, respectively) (Table 3). Azide, however, did not inhibit lucigenin Cl with either stimulant. Taurine, a scavenger of hypochlorous acid, also strongly inhibited luminol CL stimulated by both zymosan (73%) and PMA (48%) at 2.5 mM (Table 3). In addition, taurine inhibited luminol CL stimulated by zymosan in a dose-dependent manner between 0.025 and 10 mM ($P < .05$) (Fig. 3). A concentration of 1 mM caused 50% inhibition, while a 5

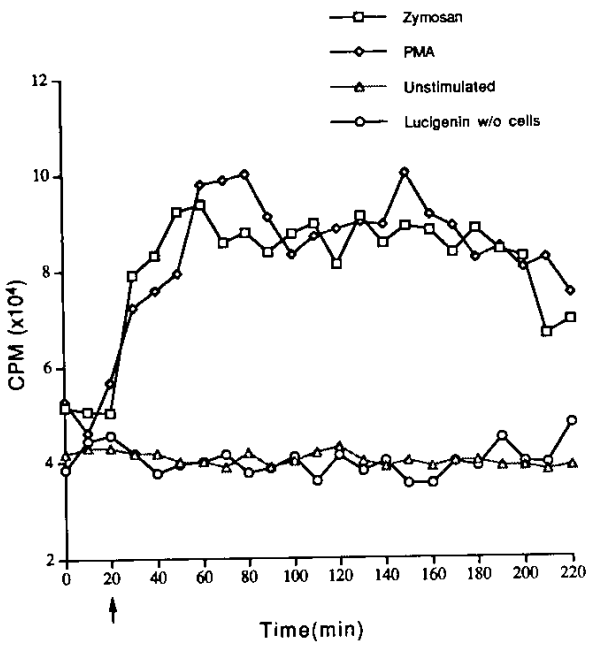

Fig. 2. Lucigenin-dependent chemiluminescence produced by oyster hemocytes at rest and following stimulation with either zymosan or PMA indicated by the arrow. There was no difference in the magnitude of the total lucigenin CL produced following stimulation with either PMA or zymosan.

mM or greater concentration caused 100% inhibition. Taurine had no effect on lucigenin CL with either stimulant (Table 3).

Neither catalase nor mannitol, scavengers of hydrogen peroxide and hydroxyl ion, respectively, had an inhibitory effect on any of the CL responses (Table 4). In fact, mannitol significantly enhanced all the CL responses except lucigenin CL stimulated by zymosan. None of the ROI scavengers tested affected CL in the presence of either probe without cells, or with cells alone.

Relationship between CL and Perkinsus marinus *infection intensity*

Luminol CL was significantly ($P = .0007$; three-factor ANOVA among probe, stimulant, and infection intensity) enhanced at the highest infection intensity regardless of the stimulant employed (Fig. 4). However, there were no significant differences in lucigenin CL with either stimulant related to infection intensity.

Since the scavenger and inhibitor experiments were done with hemocytes from oysters of different infection levels, the effects of the treatments on CL might be dependent on infection intensity. However, there was no significant interaction between the different treatments and infection. The effects of the scavengers/inhibitors on CL were statistically the same regardless of infection intensity. Therefore, significant changes in the CL of hemocytes exposed to scavengers/inhibitors were due exclusively to the treatments and not infection intensity.

Concentration of taurine in oyster tissues

The concentration of taurine in the hemocytes was much lower than that found in gill tissue. The average concentration in gill tissue was 37 nmoles/mg protein (150 μmoles/g dry wt.), whereas the hemocytes contained 3 nmoles/mg protein. The average concentration of taurine present in the cell-free hemolymph was 2 nmoles/mg protein or 360 μmoles/liter hemolymph. None of the oysters from which the taurine concentrations were measured were infected with *P. marinus*.

DISCUSSION

The results of this study suggest that hypochlorous acid (HOCl) was the primary reactive oxygen intermediate (ROI) responsible for the chemiluminescence (CL) produced by oyster hemocytes in response to phagocytic stimulation. The complete

TABLE 2. The effects of SOD on luminol and lucigenin chemiluminescence produced by oyster hemocytes stimulated with either zymosan or PMA[1]

Scavenger	N	LUM+ZYM	LUM+PMA	LUC+ZYM	LUC+PMA
Control	9	100	100	100	100
SOD 600 U/ml	2	100 (2.5)	94 (8.0)	82 (9.0)	75 (2.9)**
SOD 1,200 U/ml	3	95 (0.3)**	86 (4.6)*	109 (3.8)	70 (6.4)*
SOD 2,400 U/ml	4	64 (8.2)*	77 (2.5)*	32 (3.5)*	65 (3.6)**

[1]Nos. presented are the % of the control response (100%). Statistical significance was determined by using a single sample t-test with the population mean (m) equal to 100 (StatView II; Abacus Concepts, Inc.; Berkeley, CA). $P \leq .05$ was considered significant.
Nos. in parentheses are SEM.
*$P \leq .05$.
**$P < .01$.

TABLE 3. The effects of azide and taurine on luminol and lucigenin chemiluminescence produced by oyster hemocytes stimulated with either zymosan or PMA[1]

Scavenger	N	LUM+ZYM	LUM+PMA	LUC+ZYM	LUC+PMA
Control	10	100	100	100	100
Azide 10 μM	2	60 (1.1)**	40 (1.1)**	80 (23.9)	171 (28.6)
Azide 100 μM	5	20 (9.7)**	34 (11.2)**	119 (12.2)	96 (9.7)
Taurine 2.5 mM	3	27 (1.0)**	52 (1.5)*	100 (0.9)	85 (12.6)

[1]Nos. presented are the % of the control response (100%). Statistical significance was determined by using a single sample t-test with the population mean (m) equal to 100 (StatView II; Abacus Concepts, Inc.; Berkeley, CA). $P \leq .05$ was considered significant.
Nos. in parentheses are SEM.
*$P \leq .05$.
**$P \leq .01$.

inhibition of luminol CL by taurine indicated that the CL generated by hemocytes following stimulation with zymosan was directly dependent on HOCl production. These results agree with studies of vertebrate phagocytes in which taurine also completely inhibited luminol CL, demonstrating that HOCl was the major ROI responsible for the CL produced by these cells (Brestel, '85, '87; Gyllenhammar, '89). Superoxide dismutase partially inhibited the CL of oyster hemocytes, indicating that superoxide anions were also produced during phagocytosis and contributed to the generation of CL. Similarly, SOD reduced the luminol CL of *Lymnaea stagnalis* hemocytes by 70% during phagocytosis of zymosan granules (Adema et al., '91b) and inhibited a small portion of the luminol CL produced by human leukocytes exposed to zymosan (Stevens et al., '78; Allen, '86). The failure of catalase and mannitol to inhibit CL indicated that neither hydrogen peroxide nor hydroxyl radicals were directly involved in CL production.

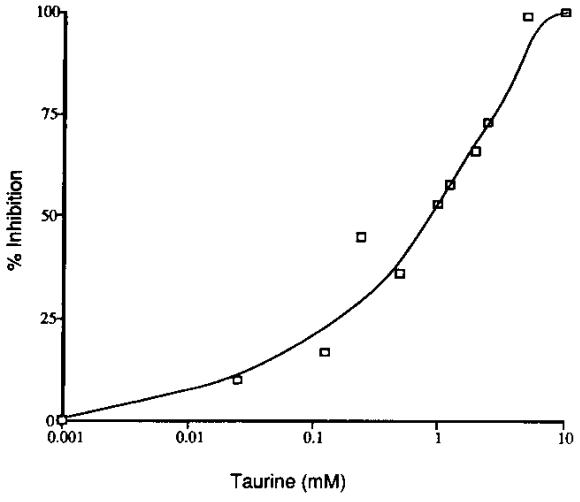

Fig. 3. Taurine inhibition of luminol-dependent chemiluminescence of oyster hemocytes stimulated with zymosan.

The azide inhibition of the luminol CL of oyster hemocytes stimulated with zymosan suggests that myeloperoxidase was directly involved in the production of HOCl during phagocytosis. These results support earlier hypotheses that the luminol CL of oyster hemocytes primarily measures the activity of the myeloperoxidase/hydrogen peroxide (MPO/H_2O_2) system, which is responsible for the generation of HOCl (Anderson, '94). Azide is oxidized by MPO in the presence of H_2O_2, resulting in the formation of nitric oxide which tightly binds to the heme component of MPO and causes inactivation of the enzyme (Nicholls, '64; Ohno and Gallin, '85). However, the results of the azide experiments in this study must be viewed with some caution since azide is a very non-specific enzyme inhibitor that effects a diverse array of cellular functions, including oxygen consumption, ATP hydrolysis, and cell volume regulation (Daggett et al., '85; Jault et al., '89; Kidder and Awayda, '89; Larson and Jagendorf, '89; Zhang and Tyerman, '91). Azide also inhibits the activity of other heme enzymes besides MPO such as catalase (Nicholls, '64; Ohno and Gallin, '85). Furthermore, azide is a scavenger of $^1O_2$; thus its effects on the luminol CL produced by oyster hemocytes could indicate the production of $^1O_2$ by these cells (Sagone et al., '77; Allen, '86; Campbell, '88). However, the taurine inhibition of luminol CL indicated that HOCl, rather than $^1O_2$, was produced by oyster hemocytes during phagocytosis. In addition, azide did not affect superoxide production of oyster hemocytes as measured by lucigenin CL. Therefore, despite the non-specific nature of azide, the combined results of the taurine and azide treatments strongly indicate the involvement of MPO in the production of HOCl during phagocytosis and the resulting luminol CL.

The luminol CL produced by hemocytes stimu-

TABLE 4. The effects of mannitol and catalase on luminol and lucigenin chemiluminescence produced by oyster hemocytes stimulated with either zymosan or PMA[1]

Scavenger	N	LUM+ZYM	LUM+PMA	LUC+ZYM	LUC+PMA
Control	7	100	100	100	100
Mannitol 1mM	4	108 (0.5)**	210 (35.4)*	272 (104.2)	187 (30.7)*
Catalase 500 U/ml	3	104 (20.5)	246 (149.0)	119 (12.1)	118 (9.1)

[1]Nos. presented are the % of the control response (100%). Statistical significance was determined by using a single sample t-test with the population mean (m) equal to 100 (StatView II; Abacus Concepts, Inc.; Berkeley, CA). $P \leq .05$ was considered significant.
Nos. in parentheses are SEM.
*$P \leq .05$.
**$P \leq .01$.

lated with PMA was also inhibited by azide and taurine. This suggests that in addition to zymosan stimulation, the CL induced by PMA may also be associated with MPO activity and the production of HOCl. The luminol CL produced by both human monocytes and PMNs stimulated with PMA is dependent on MPO activity (Briheim et al., '84; Allen, '86; Johansson and Dahlgren, '89). Whereas stimulation with zymosan triggers phagocytosis, PMA is thought to specifically activate NADPH oxidase, resulting in the production of superoxide anions (Nakagawara et al., '84; Ohtsuka et al., '90). However, SOD only partially inhibited the luminol CL of PMA-stimulated hemocytes, suggesting that PMA induces the production of other ROI species in addition to superoxide anions. Enhanced superoxide production in hemocytes stimulated by PMA may lead to increased hydrogen peroxide production, which would provide substrate for MPO activity. Therefore, the luminol CL of PMA-stimulated oyster hemocytes likely represents HOCl production.

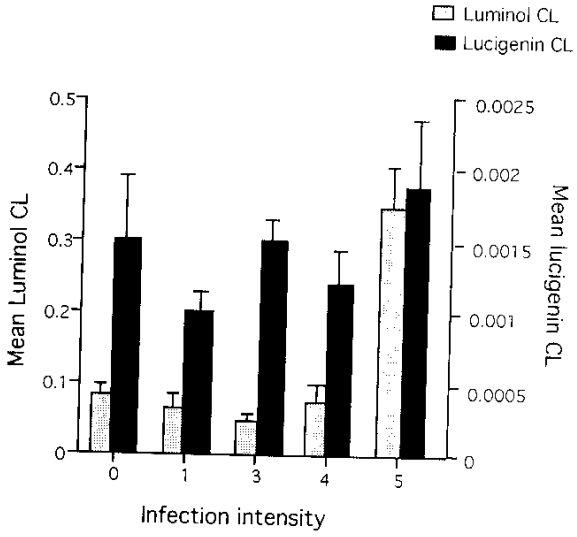

Fig. 4. The relationship between chemiluminescence and *Perkinsus marinus* infection intensity. Luminol CL was significantly enhanced at the highest infection intensity, whereas lucigenin CL was not affected by infection intensity. The magnitude of luminol CL is 100 times higher than that of lucigenin CL. Mean CL is expressed as ln(1 + x). Difference sin the CL responses associated with infection intensity were assessed by a three-factor ANOVA among probe, stimulant, and infection intensity on the log transformed data. No differences existed between stimulants so data for both were pooled to generate single mean values for each infection intensity. Error bars represent 1 standard deviation.

The failure of catalase, a scavenger of H_2O_2, to inhibit the CL produced by hemocytes during phagocytosis of zymosan was surprising. Since H_2O_2 is a substrate in the MPO reaction that produced HOCl, catalase should have interfered with the formation of HOCl. However, large molecular weight scavengers such as catalase and SOD may not easily enter oyster hemocytes and gain access to intracellular sites of ROI production. The addition of SOD, catalase, or human serum albumin to human monocytes and PMNs only partially reduces luminol CL, suggesting that CL arises from both intracellular and extracellular reactions (Briheim et al., '84; Allen, '86; Dahlgren et al., '89; Johansson and Dahlgren, '89). Therefore, sufficient catalase may not have entered the oyster hemocytes during phagocytosis to scavenge the H_2O_2 produced intracellularly, thus allowing for the production of HOCl. In addition, the activation of MPO in vertebrate phagocytes is associated with a drop in phagosome pH from neutral to approximately 4.8 (Zgliczynski et al., '71; Bakkenist et al., '80; Allen, '86), which is close to the pH optimum of 4.5 for MPO activity (Nicholls, '64; Bakkenist et al., '80; Ohno and Gallin, '85). This low pH in the phagosome may inactivate catalase, which has a more neutral pH optimum (Aebi, '83), thus preventing H_2O_2 degradation. The similar pH change in phagosomes of oys-

ter hemocytes could account for the catalase results; however, the acidification of the phagosome lumen in molluscan blood cells has not been demonstrated.

The lucigenin CL of hemocytes stimulated by either PMA or zymosan was inhibited by SOD, indicating that lucigenin detected the production of superoxide anions. Since azide did not inhibit lucigenin CL, the ROI production detected by lucigenin was probably not associated with MPO activity. Interestingly, SOD only caused a 35% inhibition of lucigenin CL in PMA-stimulated oyster hemocytes, whereas it completely inhibited lucigenin CL in human leukocytes stimulated with PMA at lower SOD concentrations than those used in this study (Allen, '86). It is possible that the concentrations of SOD tested here were not sufficient to scavenge the entire amount of superoxides produced by oyster hemocytes following stimulation with PMA or that only extracellular superoxide production was inhibited. The portion of the superoxide production detected by lucigenin may be of intracellular origin, which may not be affected by extracellular SOD. Intracellular superoxide production in oyster hemocytes was previously measured by nitroblue tetrazolium reduction assays (Anderson et al., '92b; Anderson, '94). In addition, the lucigenin CL generated by alveolar macrophages, rat liver microsomes, and aerobic bacteria also resulted from intracellular superoxide production and was not inhibited by the addition of SOD (Esterline and Trush, '89; Ischiropoulos et al., '89; Peters et al., '90). Last, stimulation of oyster hemocytes with PMA may result in the production of other ROI species which are detected by lucigenin. Although lucigenin is reported to selectively react with superoxide anions, the ambiguous results obtained here suggest that lucigenin may not be a selective probe in all cases.

Luminol CL was significantly enhanced in hemocytes from oysters heavily infected with the parasite *Perkinsus marinus*. Since luminol CL was dependent on HOCl production, these results suggest that hemocytes from infected oysters produced increased quantities of HOCl. Therefore, infection in oysters may induce increased production of HOCl by the hemocytes as a defense response. The elevated HOCl production may be the result of changes in the activity of ROI-generating enzymes triggered by infection. It is possible that infection leads to increased MPO activity, which would account for enhanced HOCl production.

Finally, the concentration of taurine found in the oyster hemolymph was within the range of concentrations that inhibited the luminol CL produced by hemocytes during phagocytosis of zymosan. A taurine concentration of 360 μmoles/liter, the amount measured in the hemolymph, produced a 25–30% inhibition of CL in vitro. This suggests that the amount of taurine normally present in the hemolymph of oysters is sufficient to scavenge significant amounts of HOCl generated by the hemocytes. Therefore, extracellular taurine may reduce the effectiveness of the hemocyte defense response to infections. Alternatively, the taurine located intracellularly may serve to protect oyster tissues from oxidant-induced injury mediated by the MPO-H_2O_2-chloride system as reported in other species (Zgliczynski et al., '71; Weiss et al., '82; Brestel, '85). Paynter et al. ('95) have shown that taurine concentrations in oyster gill tissues are reduced by *P. marinus* infection. It is possible that infection results in the loss of intracellular taurine from gills and other tissues into the hemolymph or extracellular fluid. Higher amounts of taurine in the hemolymph and extracellular fluid may then quench the effectiveness of the hemocytic oxidative immune response. The changes in gill taurine concentrations induced by infection and the role that taurine may play on quenching HOCl produced by blood cells may be related to the susceptibility or resistance of different species to this parasite.

In conclusion, the results reported here suggest that the pathway of ROI production in oyster hemocytes during phagocytosis ends in the formation of HOCl which is primarily responsible for the production of luminol CL. Infections like *P. marinus* appear to induce hemocytes to produce increased quantities of HOCl as a defense response. Last, extracellular taurine may mitigate the effectiveness of HOCl production as a mechanism of defense against such infections.

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