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Anderson, RS; Paynter, KY; and Burreson, EM, "Increased Reactive Oxygen Intermediate Production by Hemocytes Withdrawn from *Crassostrea virginica* Infected with *Perkinsus marinus*" (1992). *VIMS Articles*. 1698.

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Increased Reactive Oxygen Intermediate Production by Hemocytes Withdrawn from *Crassostrea virginica* Infected with *Perkinsus marinus*

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Abstract. *Perkinsus marinus* is a protozoan parasite responsible for a major infectious disease of the Eastern oyster, *Crassostrea virginica*. Nonspecific immunity was assayed in oysters with known intensities of infection so that the physiological responses of the host elicited by the parasite could be better understood. This report describes the capacity of hemocytes to generate reactive oxygen intermediates during the progression of the disease. The hemocytes constitute the major internal defense effector system of oysters, and cytotoxic oxygen species are thought to play central roles in antimicrobial activities of hemocytes and other phagocytic cells. Production of oxyradicals by both resting and phagocytically stimulated hemocytes was quantified by luminol-augmented chemiluminescence. Hemocytes from oysters with heavy *Perkinsus* infections produced significantly higher levels of chemiluminescence than their counterparts withdrawn from lightly or moderately infected individuals. Furthermore, in addition to a higher chemiluminescent activity per cell, the total circulating hemocyte count was elevated in the heavily infected animals. Therefore, advanced cases of this disease seem to be characterized by hemocyte activation and recruitment, with concomitant exuberant production of hemocyte-derived reactive oxygen intermediates. The resultant oxidant load may participate in the pathogenesis of the disease.

Introduction

Upon appropriate stimulation, phagocytic cells typically undergo a burst of respiratory activity and manufacture large quantities of cytotoxic oxidants. This activity was first reported in mammalian neutrophils and macrophages, but has subsequently been observed in phagocytes from most vertebrates and invertebrates. The details of the production of reactive oxygen intermediates (ROIs) by these cells may be found in reviews by Badwey and Karnovsky (1980), DeChatelet *et al.* (1982), Klebanoff (1982), Babior (1984), and Babior *et al.* (1988). Cell-derived ROIs can participate in protective antimicrobial mechanisms and mediate various kinds of host tissue injury. The ultimate source of these oxidants is the superoxide anion (O_2^-) produced by the one-electron reduction of oxygen at the expense of NADPH; the reaction is catalyzed by a membrane-bound flavoprotein oxidase. Superoxide undergoes enzymatic dismutation to produce hydrogen peroxide (H_2O_2), which may give rise to other highly toxic species, such as the hydroxyl radical ($\cdot OH$), via the Fenton reaction, or hydrochlorous acid ($HOCl$) via myeloperoxidase. The roles of phagocyte-generated ROIs in the pathogenesis of certain mammalian diseases has gained considerable recent attention (*e.g.*, see Halliwell, 1988; Cerutti *et al.*, 1988) particularly with regard to inflammatory cell damage of the vascular endothelium in atherosclerosis, tissue injury during myocardial ischemia and reperfusion, immune complex-induced acute lung injury, and glomerulonephritis. The health of aquatic organisms may also be effected by oxidative stress (DiGiulio

Received 1 June 1992; accepted 14 September 1992.

Abbreviations: CL = chemiluminescence; CSM = cell supported medium; MB = Mobjack Bay; ROI = reactive oxygen intermediates.

et al., 1989). The mechanisms underlying oxidant damage of cells are numerous but are thought to include: decreased ATP levels via inhibition of mitochondrial oxidative phosphorylation, increased DNA strand breaks and other structural and functional alterations of nucleic acids, interference with intracellular enzymes, peroxidation of membrane lipids with concomitant reduction in structural integrity, and various other deleterious interactions with cell membrane proteins and lipids (Fantone and Ward, 1982; Kako, 1987; Cochrane *et al.*, 1988).

As previously noted, the respiratory burst and accompanying production of ROIs are not restricted to mammalian phagocytes. The literature on these activities by molluscan hemocytes has been expanding since the initial report of H₂O₂ release from scallop amebocytes by Nakamura *et al.* (1985). Recently, the hemocytes of many molluscan species have been shown to produce O₂⁻ and H₂O₂ (Dikkeboom *et al.*, 1987 and 1988; Adema *et al.*, 1991a; Bachère *et al.*, 1991a; Pipe, 1992), and this activity may be modulated by exposure to certain environmental contaminants (Larson *et al.*, 1989; Fisher *et al.*, 1990; Anderson *et al.*, 1992a, 1992b) or by microbial infection (LeGall *et al.*, 1989, 1991; Hervio *et al.*, 1989). Myeloperoxidase activity has also been measured in bivalve hemocytes (Schlenk *et al.*, 1991). The subject of ROI expression by molluscan phagocytes has been reviewed recently by Adema *et al.* (1991b).

As is the case for of oxyradicals in other species, molluscan ROIs probably participate in antimicrobial defense, but they also have a destructive potential. The balance between ROI production and activities of protective mechanisms (superoxide dismutase (EC 1.15.1.1), catalase (EC 1.11.1.6), glutathione peroxidase (EC 1.11.1.9), glutathione, vitamins A, C and E, β carotene, etc.) is critical in determining the extent of oxidative damage. The contribution of ROIs from activated hemocytes to oxidant loads already present due to normal or pollution-enhanced oxyradical production (reviewed by Livingstone *et al.*, 1989, 1990; Winston *et al.*, 1990; Winston, 1991) could theoretically overwhelm the antioxidant defense mechanisms of mollusks.

In this paper we report that hemocytes from oysters heavily infected with the protozoan parasite *Perkinsus marinus* produce significantly elevated levels of ROIs; the possibility that this contributes to the pathogenesis of the disease is raised.

Materials and Methods

Animals

The oysters (*Crassostrea virginica*) used in this study were spawned in July 1989 and introduced as spat into an area with low *Perkinsus* (the Wye River, MD, salinity ~eight ppt) in September 1989. Two groups from this

population were transferred to a high *Perkinsus* site in Mobjack Bay, VA (~20 ppt salinity): the first group on 14 April 1991, and the second group on 28 July 1991. Both the *Perkinsus* prevalence and the higher salinity of Mobjack Bay favored infection and progression of the disease in the transplanted oysters (Paynter and Burreson, 1991). The above transfer protocol was designed to make available oysters with a wide spectrum of disease stages.

Both groups of Mobjack Bay oysters were sampled twice (on 10 and 17 September 1991), held on ice, and transported to the laboratory within 24 h of collection. They were numbered and housed at ambient temperatures and salinity (23°C, 20 ppt) in aerated aquaria. Hemolymph samples were withdrawn from the adductor muscle sinus for chemiluminescence (CL) studies and for hemocyte enumeration; all of the hemolymph samples were collected within three days of receipt of the oysters. The animals were replaced in the aquaria; all specimens living at the end of the hemolymph collection period (three days) were individually diagnosed and staged for *Perkinsus* by the thioglycollate method (Ray, 1952, 1966).

Oysters that became moribund before they could be diagnosed by the thioglycollate method were fixed in Davidson's AFA and examined by histology. Histological diagnoses have been found to provide slightly lower estimates of infection levels than the thioglycollate method. Based on this experience, the histological diagnoses were adjusted slightly to enable them to be expressed by the thioglycollate scoring method. In this study, 55 diagnoses were carried out via thioglycollate, and 25 were initially performed by histology and converted to thioglycollate scores. To quantify the level of infection in individual oysters, the subjective criteria of light, moderate, and heavy, based on Ray (1952), were given the numeric values of 1, 3, and 5, respectively.

Reactive oxygen intermediate determination

The production of ROIs by oyster hemocytes was quantified by measuring luminol-augmented chemiluminescence (CL) before and after phagocytic stimulation. To obtain the hemocytes, several ml of hemolymph were withdrawn from the adductor muscle sinus via a small notch cut in the edge of the valves. An aliquot was placed on a hemacytometer and the total cell count determined; the remaining hemolymph was further processed for CL evaluation. The hemolymph was incubated for 15 min at room temperature in a plastic petri dish, during which time the majority of the cells became firmly attached. Filter-sterilized ambient water from the oyster tanks (FA) was then used to gently wash off the fluid phase of the hemolymph and any unadhered cells. The cell monolayer was then overlaid with FA and incubated for 2.5 h at room temperature. During this period the cells became

progressively more loosely attached and could be finally washed free and resuspended in FA. These cells retained phagocytic activity, but showed little of the clumping typically encountered during the centrifugation and resuspension of freshly collected cells. The hemocytes were removed from suspension by centrifugation (200 g, 15 min), resuspended in cell support medium (CSM), and counted. The composition of CSM was 0.5% antibiotic solution (10,000 U penicillin, 10 mg streptomycin, and 25 µg amphotericin B/ml), 5% fetal calf serum, and 1 mg/ml glucose in FA. One ml aliquots of the hemocyte suspension (10^6 cells in CSM) were placed in small scintillation vials. From this point on, all steps were carried out in a dark room under dim red illumination. To each vial was added 0.3 ml FA and 1 ml luminol (250 µM in FA), and the vials were placed in a scintillation counter programmed for single photon monitoring. The vials were counted at 0.3 min intervals for about 10 min to establish the level of unstimulated, background CL for each sample. Then 0.2 ml of a suspension of heat-killed, washed yeast cells (final particle:hemocyte ratio = ~20:1) were added to the vials to provide phagocytic stimulation. The vials were counted at 0.3 min intervals for about 2 h after the addition of yeast.

Chemiluminescence (counts per min) was recorded directly on computer disks and as printed output from the scintillation counter. The background (basal) CL level was defined as the cpm recorded immediately before the addition of the phagocytic stimulus. The peak CL was the maximal CL response elicited by the phagocytic event (corrected for background). The total CL response was expressed as the area under the phagocytically induced portion of the curve (estimated by a polygon summation method).

The raw CL data were not normally distributed and were transformed ($\ln [1 + X]$) to permit statistical analysis of variance; multifactor ANOVA was performed as appropriate. Differences between data groups were considered significant if $P < 0.05$ (F-test). Post hoc tests (Fisher PLSD and Scheffe F-test) were also run and significance at the 95% level determined.

Results

Batch differences

Two batches of oysters, taken a week apart, were sampled from each of the two transplanted groups in Mobjack Bay. The possibility that the two batches were significantly different from each other with regard to mean levels of CL activity was investigated. The level of infection showed no significant difference between batches within the groups. The various raw CL parameters showed a trend (batch 1 < batch 2); after log transformation, the only parameter to show this effect was total CL ($P = 0.0298$).

Table I

Intensities of *Perkinsus marinus* infection in oyster groups with different residency times in Mobjack Bay (MB)

Infection level*		
Group 1 (7 wks in MB; n = 39)	Group 2 (22 wks in MB; n = 41)	P value ($\ln [1 + X]$ transformed data; F-test, one factor ANOVA)
1.6 ± 0.2	3.8 ± 0.3	0.0001

* Each oyster scored for level of infection: 1 = light, 3 = moderate, 5 = heavy.

Because of the highly significant differences reported below between the two transplanted groups, with regard to all CL parameters and infection levels, the batch effects were considered minimal, and the data were analyzed by two factor ANOVA using group data and levels of infection.

Group differences

As outlined in Materials and Methods, the two groups were derived from the same spawning, but differed with regard to the period spent in Mobjack Bay (MB), during which time they were exposed to increased salinity, increased *Perkinsus* exposure, and other environmental factors present in that location. As expected, the mean level of infection recorded for group 2 (~22 weeks in MB) was higher than that of group 1 (~7 weeks in MB), as seen in Table 1. Table IIa indicates that the background,

Table II

Luminol-augmented chemiluminescence by 10^6 hemocytes from oysters infected with *Perkinsus marinus*

a: Hemocytic chemiluminescence (mean cpm ± SEM) from oyster groups with different intensities of *Perkinsus marinus* infection.

CL parameter	Group 1 Lightly infected (n = 39)	Group 2 Heavily infected (n = 41)	P value (F-test, two factor ANOVA, $\ln [1 + X]$ transformed data)
Background	121 ± 22	347 ± 49	0.0294
Peak	276 ± 107	1,050 ± 174	0.0031
Total	23,632 ± 9,266	80,720 ± 13,513	0.0098

b: Hemocytic chemiluminescence (mean cpm ± SEM) from individual oysters with various levels of *P. marinus* infection.

CL parameter	Level of infections			P value (calculated as above)
	Light (n = 38)	Moderate (n = 13)	Heavy (n = 28)	
Background	128 ± 20	105 ± 22	452 ± 62	0.0115
Peak	305 ± 105	253 ± 85	1,388 ± 229	0.0036
Total	24,834 ± 8,336	18,506 ± 6,499	108,771 ± 18,265	0.0057

peak, and total CL parameters of the more heavily infected group were significantly greater than the comparable values of the lightly infected group. The Fisher PLSD and Scheffe F-tests were also significant at the 95% level when the CL parameters were compared between groups.

Correlation of CL parameters with levels of infection in individuals

Since groups differed with regard to average levels of infection, and the CL responses seemed to be related to the levels of infection, we asked whether infection intensities were directly correlated with magnitude of CL responses in individual oysters. Diagnostic data and CL values were available for each oyster, and these data were analyzed for individuals without consideration of the particular groups from which they were collected. Table IIb shows that there were significant differences between mean CL parameters in oysters with light, moderate, or heavy *Perkinsus* infections. However, further examination showed that these differences were significant (95% level, Fisher PLSD and Scheffe F-test) when comparing light *versus* heavy or moderate *versus* heavy infections, but not when comparing light *versus* moderate. Significant CL response differences can apparently only be seen between oysters with heavy *Perkinsus* infections and those with less intense infections. Since none of the oysters in this study were totally *Perkinsus*-free, we do not yet know whether uninfected oysters can be differentiated from lightly or moderately infected individuals based on their CL responsiveness.

When their relative contributions to the observed variation (induction) of CL parameters were analyzed (data in Tables IIa and b compared), the effects were clearly more associated with the levels of infection than with the group. Moreover, there were no significant interactions between individual and group infection level effects ($P > 0.2$ for background, peak, and total CL).

Total hemocyte counts in Perkinsus-infected oysters

During the preparation of cells from batch 1 for CL assays, we noticed that the hemolymph from the heavily infected group sometimes seemed more turbid than that from the lightly infected group. This was tentatively ascribed to the presence of elevated numbers of circulating hemocytes; therefore, hemocyte counts were carried out on the second batch ($n = 36$). The total hemocyte counts in the second group of oysters (the more heavily infected group of Mobjack Bay transplants) were not significantly higher than those recorded for the lightly infected group (Table IIIa). But if the hemocyte data were grouped according to diagnostic scores of individual oysters (Table IIIb), the correlation between heavy intensity of infection and hemocyte numbers was noticeable, and the differences

Table III

Effects of Perkinsus marinus infection on total hemocyte count (THC) in Crassostrea virginica

a: Hemocyte numbers in lightly and heavily infected groups, as defined in Table I.

Total hemocyte count $\times 10^6$ /ml, mean \pm SEM		
Group 1 Lightly infected ($n = 18$)	Group 2 Heavily infected ($n = 18$)	<i>P</i> value ($\ln [1 + X]$ transformed data, F-test, two factor ANOVA)
2.78 \pm 0.66	4.15 \pm 0.65	0.267

b: Hemocyte numbers in individual oysters with known intensities of infection.

THC $\times 10^6$ (mean \pm SEM)	Levels of infection			<i>P</i> value (calculated as above)
	Light ($n = 20$)	Moderate ($n = 6$)	Heavy ($n = 10$)	
2.52 \pm 0.47	2.95 \pm 0.54	5.68 \pm 1.15	0.002	

between these groups were significant. Post hoc tests were also run to determine the significance of the differences observed between cell counts from oysters with different *Perkinsus* diagnostic scores. These results showed that significant differences (at the 95% level, Fisher PLSD test) were encountered when comparing light *versus* heavy and moderate *versus* heavy, but not light and moderate. This was reminiscent of the findings when chemiluminescence data were compared according to these classifications.

Discussion

The method of transferring groups of oysters into Mobjack Bay at different times successfully produced populations with significantly different average levels of *Perkinsus* infection. However, at the times of sampling, no oysters were obtained that were entirely free of the sporozoan parasite. In an attempt to understand the status of the oysters' internal defense mechanisms during the course of the infection, the chemiluminescence (CL) response of hemocytes withdrawn from these oysters was determined. We knew that reactive oxygen intermediates (ROIs) produced by hemocytes were probably important to antimicrobial defense and that luminol-augmented CL is a sensitive method of ROI quantitation. Therefore, we predicted that decreased CL might be associated with *Perkinsus* infection, either as a result of the disease, or as a component of decreased resistance to the disease. Indeed, there is evidence that such is the case for certain other infections in bivalves. For example, the parasite *Bonamia*

ostreae failed to stimulate CL production after ingestion by *Ostrea edulis* hemocytes (Hervio *et al.*, 1989; Bachère *et al.*, 1991b) and similar inhibition of CL followed phagocytosis of Rickettsiales-like organisms by the cells of *Pecten maximus* (LeGall *et al.*, 1991). But the data reported here show that established *Perkinsus* infections apparently have the opposite effect: the hemocytes produce large amounts of ROIs, both at rest and after phagocytic stimulation.

The experimental groups varied with regard to residence time in Mobjack Bay (length of exposure to elevated salinity and to any environmental contaminants that might be present), as well as with regard to average level of infection. Although the water chemistry at the Mobjack Bay site was not analyzed, the area is considered to be relatively clean. Furthermore, exposure of bivalves to environmental pollutants can result in hemocyte CL suppression (Larson *et al.*, 1989; Fisher *et al.*, 1990; Anderson *et al.*, 1992a, b), but rarely causes enhancement. Preliminary studies in this laboratory have failed to produce ROI modulation in hemocytes after salinity changes greater than that (8–20 ppt) experienced by the oysters in this study. Furthermore, since this salinity shock occurred a minimum of seven weeks before the oysters were collected; the animals probably had ample time to recover from this physiological stress. Therefore, the differences in CL responses observed between the groups are probably due to differences in *Perkinsus* infection intensities.

The relationship between infection and CL activity was further clarified when considered for individually diagnosed animals without regard to experimental group designations. Statistical analysis of the data showed that both resting and induced CL parameters in oysters with heavy infections were elevated compared to oysters with light or moderate infections. Clearly, as the disease progressed, the circulating hemocytes produced greater amounts of ROIs and developed an increased capacity for ROI release upon phagocytic stimulation. In short, these cells appear to be activated, perhaps as a result of endocytosis of the *Perkinsus* organism itself; the sporozoan may be resistant to intracellular destruction and appears to be disseminated within the host via transport in hemocytes.

Although the mechanism of increased CL responsiveness of hemocytes from *Perkinsus*-infected oysters is not known, the phenomenon seems to be established. Preliminary results reported here also indicate that the total hemocyte count per ml hemolymph is higher in oysters with heavy infections. These two factors could work together to generate oxidant loads that could produce various forms of inflammatory cell damage in oysters during the later stages of infection, and play a role in the pathogenesis of the disease.

Acknowledgments

Expert technical assistance was provided by Laurie M. Mora, Lisa L. Brubacher, and Juanita Walker. This study was funded in part by NOAA Grant NA16FL0400–01.

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