Pergamon

Developmental and Comparative Immunology, Vol. 19, No. 2, pp. 135–142, 1995

0145-305X/95 $9.50 + .00

0145-305X(94)00059-X

SUPPRESSION OF CHEMILUMINESCENCE OF EASTERN OYSTER (*Crassostrea virginica*) HEMOCYTES BY THE PROTOZOAN PARASITE *Perkinsus marinus*

Aswani K. Volety and Fu-Lin E. Chu

School of Marine Science, Virginia Institute of Marine Science, College of William & Mary, Gloucester Point, VA 23062
(Submitted May 1994; Accepted December 1994)

□Abstract—Experiments were conducted to determine the ability of the protistan parasite, *Perkinsus marinus*, to inhibit chemiluminescence of hemocytes from the eastern oyster, *Crassostrea virginica*. Luminol-enhanced chemiluminescence (CL) was used to measure the production of reactive oxygen intermediates (ROI) generated by oyster hemocytes using zymosan as a stimulant. To determine whether *P. marinus* suppresses ROI evoked from zymosan-stimulated hemocytes, live or heat killed *P. marinus* in filtered estuarine water (*YRW*) (salinity = 20 ppt) were added to (1) zymosan-stimulated hemocytes after CL reached its peak, or (2) hemocytes at the same time as zymosan, and reduction of CL responses were recorded. In both tests, controls received only estuarine water. Live *P. marinus* meronts significantly suppressed ROI production by zymosan-stimulated hemocytes. The suppression of ROI production was dose dependent. Suppression of ROI production from zymosan-stimulated hemocytes by heat killed *P. marinus* was significantly less than by live *P. marinus*. Similarly, CL of hemocytes was reduced, though not significantly when hemocytes were exposed to YRW preincubated with *P. marinus*. When *P. marinus* meronts were used as a stimulant, no CL response was elicited. Results of this study suggest that *P. marinus* cells are able to suppress ROI release from oyster hemocytes, thus evading this component of the host's defense.

□Keywords—*Crassostrea virginica; Perkinsus marinus;* Hemocytes; Chemiluminescence; Reactive oxygen intermediates; Acid phosphatase; Suppression; Defense.

Address correspondence to Fu-Lin E. Chu.

Nomenclature

CL	chemiluminescence
HKP	heat killed *P. marinus*
LP	live *P. marinus*
PMYRW	*P. marinus* incubated York River water
RLO	Rickettsiales-like organisms
ROI	reactive oxygen intermediates
YRW	filtered estuarine water (York River water)
ZYM	zymosan

Introduction

Heavy mortalities of oysters in Chesapeake Bay and on the east coast of the United States caused by the protozoan parasite, *Perkinsus marinus*, have been well documented (1,2) in recent years. Although the physiopathologic effects of the disease organism on the oysters have been studied extensively (3–5), very little is known about the evasive mechanisms of the parasite in escaping the host's defense mechanisms.

Defense mechanisms in vertebrate phagocytes involve complex processes including the production of toxic free oxygen radicals by the respiratory burst (6), and other enzymatic processes (7). When vertebrate macrophage membranes are stimulated by foreign parti-

cles or organisms, stimulation of NADPH oxidase (8,9) and activation of the hexose monophosphate pathway occurs. This process is accompanied by production of toxic reactive oxygen intermediates (ROI) such as OH^-, H_2O_2, $^1O_2$, and O_2^-, which may be involved in cellular killing. Also, H_2O_2, along with myeloperoxidase and halide ions, results in the formation of hypohalites and singlet oxygen, which are microbicidal (10,11). Similar mechanisms were noted in invertebrates such as the shore crab, *Carcinus maenas* (12), sea urchin, *Strongylocentrotus nudus* (13) and molluscs, *Patinopecten yessoensis* (14), *Lymnea stagnalis* (15), *Planorborius corneus, Helix aspera* (16), *Biomphalaria glabrata* (17), *Pecten maximus* (18), *Crassostrea gigas* and *Ostrea edulis* (19), and *Crassostrea virginica* (20–22). The microbicidal activity associated with the production of ROI has been discussed by many authors (6,23–26). Release of ROI production coincided with phagocytosis of zymosan particles and chemiluminescence activity in *Lymnaea stagnalis* (23), suggesting an association between phagocytosis, ROI production, and chemiluminescence. Production of both ROI and hydrogen peroxide inside the phagosomes of *L. stagnalis* hemocytes has also been documented (15). Luminol-enhanced CL has been used to measure ROI production related to phagocytic activity in oyster species, *C. gigas* and *O. edulis* (19) and *C. virginica* (21,22). Involvement of the myeloperoxidase system in the production of ROI has been demonstrated in the common mussel, *Mytilus edulis* (11) and in *C. virginica* (Austin and Paynter, personal communication, November 1994).

Although invertebrates lack a complex immune system like that of vertebrates, they possess an effective defense system comprising cellular (27,28) and humoral activities (29). Hemocytes comprise a primary line of defense in molluscs and are responsible for activities such as inflammation, wound repair (27), phagocytosis, and encapsulation (27,30). Phagocytosis and degradation of *P. marinus* meronts have been demonstrated using transmission electron microscopy (31,32). Stimulation of oyster hemocytes with *P. marinus* meronts, however, did not elicit any CL response (31, Chu and Volety, unpublished results). This suggests that either *P. marinus* meronts are degraded by processes not mediated by ROI and/or *P. marinus* may be able to suppress, inhibit, or scavenge the ROI released by the host hemocyte. Therefore, this study was conducted to investigate the possible suppression or inhibition of the host's ROI production by *P. marinus*.

Materials and Methods

Oysters and Hemolymph Collection

Hemolymph was withdrawn from oysters (2.5–3″) collected from the Rappahannock River, Virginia. One mL of hemolymph was collected from the adductor muscle of individual oysters using a 27-gauge needle. Hemolymph was pooled and hemocyte concentration in the hemolymph was adjusted to 1×10^6 cells/mL for all CL assays in this study.

P. marinus

P. marinus meronts were cultured according to Gauthier and Vasta (33). The parasite has been subcultured for over 30 generations in our laboratory. The parasites were washed twice and resuspended in 1 μm filtered estuarine water (York River Water, YRW) at a concentration of 60×10^6 cells/mL. For the dose–response study, *P. marinus* cells were adjusted to the appropriate concentrations using YRW. Both live and heat-killed (100°C for 15 minutes) *P. marinus* were washed and resuspended in YRW at the aforementioned concentration.

Zymosan

Zymosan (Sigma, USA) particles were used as a stimulant for the oyster hemocytes. Zymosan particles were suspended in YRW at a concentration of 10 mg/mL, heated for 30 min at 100°C, washed twice, and resuspended in YRW at a concentration of 1 mg/mL.

Chemiluminescence Assay

The general procedure for Chemiluminescence (CL) measurement is as follows: CL (counts per minute, CPM) was measured in a Beckman LS-3133T liquid scintillation counter in an out-of-coincidence mode at room temperature (22–23°C). Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, 1:500 dilution) was prepared according to Scott and Klesius (34). Five hundred microliters hemolymph samples (0.5×10^6 hemocytes/sample, $N = 3$–4/treatment) and 500 μL luminol in YRW were aliquoted into plastic scintillation vials and baseline CL (CL of unstimulated hemocytes) levels were recorded for 15–30 min (2–3 cycles). Five hundred microliters of zymosan suspension were then added to the hemocyte mixture and CL responses recorded. Controls received 500 μL of YRW containing no zymosan. CL counts were plotted against time, and the total CL response was expressed as the area under the CL curve induced by the addition of zymosan/*P. marinus* from each assay. The CL area was integrated using a Numonics 2400 digitizer and expressed as CPM.

Experiments

1. *Dose-related response of hemocyte CL to* P. marinus. *P. marinus* cells were adjusted to a concentration of 7.5, 15, 30, and 60×10^6 cells/mL in YRW (hemocyte: *P. marinus* = 1:7.5, 1:15, 1:30, and 1:60). Five hundred microliters of hemolymph and 500 μL luminol solution (triplicate samples were analyzed for each *P. marinus* concentration and control) were placed in plastic scintillation vials, and baseline CL was measured. Production of ROI was initiated by the addition of 500 μL of zymosan (500 μg) and 500 μL of *P. marinus* (3.75, 7.5, 15, or 30×10^6 cells) suspensions. For each sample, counts were measured for 2–3 h (8–12 cycles, 15 min/cycle).

2. *Suppression of CL by* P. marinus. The dose of *P. marinus* (hemocyte: *P. marinus* = 1:60) that produced the most effective suppression of CL was used in this assay. Five hundred microliters of hemocyte and luminol mixture were aliquoted into scintillation vials, and the CL baseline activity was measured for 2–3 cycles (8 min/cycle). Five hundred microliters of zymosan was then added to the hemocyte mixture. When CL response reached its peak, 0.5 mL of a live ($N = 4$) or heat-killed ($N = 4$) *P. marinus* meront suspension (30×10^6 cells) was added to the zymosan-stimulated hemocytes and reduction of CL activity was measured. Counts were conducted on each sample for 2–2.5 h (15–18 cycles, 8 min/cycle). Zymosan-stimulated hemocytes ($N = 4$) with 500 μL of YRW added served as *non-P. marinus* controls. The second control (blank) received only 500 μL of YRW.

3. *Inhibition of CL by* P. marinus. In a manner similar to experiment 2 above, the same concentrations of hemocytes, parasites, and zymosan were used, with the exception that zymosan and *P. marinus* were added to the hemocyte suspension at the same time.

4. *Effect of* P. marinus *secretions on hemocyte CL.* Cultured meronts were washed twice with YRW under sterile conditions, resuspended in YRW at a cell density of 60×10^6 cells/mL ($N =$

6), and incubated at 25°C for 48 h. The cell suspensions were then centrifuged at 12000 × *g* for 10 minutes and the supernatants (PMYRW) saved. Hemocytes in 500 μL plasma were exposed to 500 μL of PMYRW. Controls were exposed to YRW. Zymosan was added to the hemocyte suspensions immediately following the addition of YRW on PMYRW, and CL responses were recorded. The second control (blank) received YRW. Acid phosphatase activity of the PMYRW and YRW was assayed using a colorimetric assay (Sigma Diagnostics) based on the release of *p*-nitrophenol and inorganic phosphate from *p*-nitrophenyl phosphate by the enzyme (35).

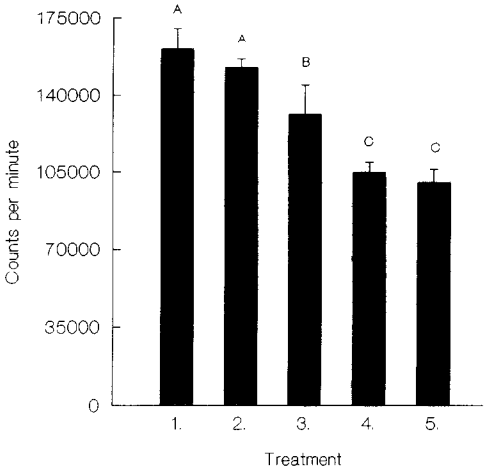

Figure 1. Dose response of oyster hemocyte CL to *P. marinus* meronts. Treatments 1, 2, 3, 4, and 5 = YRW, 3.75, 7.5, 15, and 30 × 10^6 *P. marinus* cells/5 × 10^5 hemocytes. Mean CPM of triplicate samples ± 1 SE. The same letters above the bars denote lack of significance ($p > 0.05$).

Statistical Analyses

To compare the areas under the CL curves between treatments of (i) Zymosan-stimulated hemocytes exposed to different doses of *P. marinus* and (ii) heat-killed or live *P. marinus*-exposed and control hemocytes in both suppression and inhibition assays, one-way analysis of variance was used. Paired *t* test was used to determine the differences between PMYRW- and YRW-incubated hemocyte CL and acid phosphatase activities in PMYRW and YRW. Differences were considered statistically significant if $p < 0.05$. All the experiments were repeated 3–4 times to determine the reproducibility.

Results

Since the results of all of the experiments showed a similar trend, the typical results of individual experiments are reported here.

P. marinus suppressed the CL activity of oyster hemocytes in a dose-dependent manner (Fig. 1). No significant reduction in CL was observed compared to controls when hemocytes were exposed to 3.75 × 10^6 *P. marinus* cells. However, suppression of the CL response was significant when hemocytes were exposed to 7.5, 15, and 30 × 10^6 *P. marinus* cells.

The reduction of CL was significantly higher ($p < 0.05$) in hemocytes exposed to live *P. marinus* as compared to heat-killed *P. marinus* or controls (Fig. 2) when *P. marinus* cells were added to the hemocytes at their peak CL response. However, the difference in CL response between control hemocytes and hemocytes exposed to heat-killed *P. marinus* was insignificant ($p > 0.05$).

Similar results were demonstrated when zymosan and live or heat-killed *P. marinus* were simultaneously added to the hemocytes (Fig. 3). A significant decrease in CL activity in live *P. marinus*-exposed hemocytes ($p < 0.05$) was noted, as compared to heat-killed and *non-P. marinus*-exposed controls. Again, no significant differences in CL were observed between heat-killed *P. marinus*-exposed and control hemocytes. No CL response was elicited by hemocytes when *P. marinus* meronts were used as stimulant.

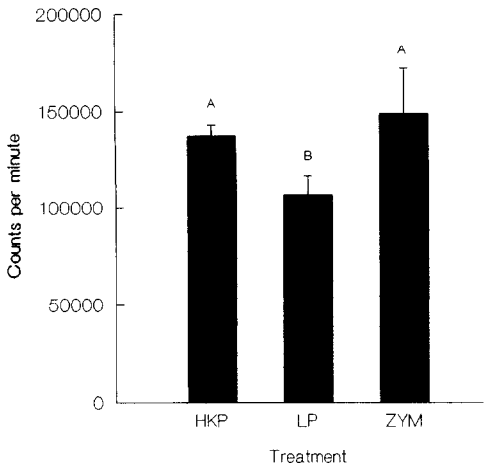

Figure 2. Suppression of zymosan-induced CL in oyster hemocytes by *P. marinus*. (HKP = heat killed-*P. marinus;* LP = live-*P. marinus,* ZYM = zymosan). *P. marinus* cells were added to hemocytes at their peak CL response. Mean CPM of four samples + 1 SE. The same letters above the bars denote lack of significance ($p > 0.05$).

The CL response (mean ± 1 SE) of hemocytes exposed to filtered YRW (control) was higher (49685 ± 1717 cpm) than that of hemocytes exposed to PMYRW (45178 ± 2266 cpm); however, the differences were statistically insignificant ($p < 0.15$). The acid phosphatase concentration (mean ± 1 SE) in PMYRW was 101 ± 10 mUnits/mL, but no acid phosphatase was detected in the controls.

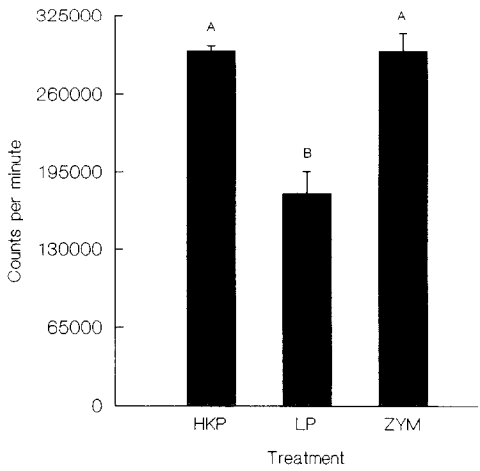

Figure 3. Inhibition of zymosan-induced CL in oyster hemocytes by *P. marinus*. (HKP = heat killed-*P. marinus;* LP = live-*P. marinus,* ZYM = zymosan). Mean CPM of four samples ± 1 SE. The same letters above the bars denote lack of significance ($p > 0.05$).

Discussion

Certain protozoan parasites employ mechanisms such as active entry into host cells, entry into the host cells without triggering the respiratory burst, and suppression and/or inhibition of ROI production by phagocytes (36–39). *Leishmania spp.* enter the host macrophage by triggering a receptor and causing internalization, but not stimulating the respiratory burst (40). Lipophosphoglycan in cell membranes of *Leishmania* promastigotes inhibits the respiratory burst of monocytes, possibly by having an inhibitory effect on protein kinase C of the host cells (41).

Electron microscopic studies by La Peyre (31) and Bushek et al. (32) indicated that the oyster hemocytes are able to recognize and phagocytose freshly isolated and laboratory cultured *P. marinus* cells, resulting in limited degradation of the parasite in the hemocytes. However, no CL response was observed from hemocytes of either American (*C. virginica*) or Pacific (*C. gigas*) oysters when exposed to *P. marinus* merozoites (31). These results suggest that intracellular killing of *P. marinus* may not be mediated by toxic metabolites. The degradation of *P. marinus* by oyster hemocytes may be mediated by enzymatic factors such as the lysosomal enzymes. Presence of lysosomal enzymes in molluscs has been documented (42–44). Presently, the exact mechanism involved in intracellular killing of *P. marinus* by oyster hemocytes remains unclear.

Results of the present study indicate that live *P. marinus* cells are able to suppress and/or inhibit ROI release from oyster hemocytes. The lack of any inhibition of ROI production by zymosan-stimulated hemocytes when exposed to

heat-killed *P. marinus* may suggest that denaturation of the ROI suppressor(s) occurred in the parasite upon heating (Figs. 2 and 3). Similar results were reported by Le Gall et al. (18) when zymosan-stimulated *Pecten maximus* hemocytes were exposed to live and heat-killed rickettsiales-like organisms (RLO). Their study demonstrated that exposure of *P. maximus* hemocytes to live RLO produced a greater suppression (45–74%) of CL activity compared to heat-killed RLO (51–58%).

Investigations of Yoshino et al. (45) revealed that the extracellular cysteine proteinase(s) of *Schistosoma mansoni* degrade high molecular weight hemolymph proteins of the host, *B. glabrata*. The YRW preincubated with *P. marinus* may contain extracellular products (e.g., acid phosphatase, catalase, superoxide dismutase, glutathione peroxidase, aminopeptidase) from the parasite responsible for suppression/inhibition of ROI production by oyster hemocytes. Antioxidant enzymes, such as superoxide dismutase, catalase (46), and glutathione peroxidase (47) were suggested to protect the parasite, *S. mansoni* from the mammalian host's respiratory burst, thus enabling the parasite to survive in the host cell. Acid phosphatase in *L. donovani* (48) and Rickettsiales-like organisms in *P. maximus* (18) reduces superoxide production by the phagocytic cells. Hervio et al. (49) suggested a similar protective role for acid phosphatase present in *Bonamia ostreae* against the host's (*O. edulis*) defense.

Preliminary studies in our laboratory using standard assay procedures failed to detect superoxide dismutase, catalase, or glutathione peroxidase in *P. marinus* meronts (unpublished results). However, relatively high concentrations of intracellular (94 mU/mg protein) and extracellular (42 mU/10^6 cells) acid phosphatase activity were observed in *P. marinus* meronts (unpublished results) when compared to host hemocytes and serum. Acid phosphatase activities in whole hemolymph, supernatant, and hemocyte pellets in *C. virginica* were 1.8, 0.7, and 47 mU/mg protein and in *Mercenaria mercenaria*, 3.5, 1.5, and 10.3 mU/mg protein, respectively (43). Our recent studies also revealed that the growth rate of *P. marinus* is higher at higher temperatures (up to 25°C). The concentration of extracellular acid phosphatase in the culture medium was positively correlated with cell number and temperature (manuscript in preparation). The possible deficiency of detectable levels of other antioxidant enzymes and the presence of acid phosphatase in *P. marinus* meronts suggest that acid phosphatase in *P. marinus* may be one of the enzymes that suppress the ROI production by the host's hemocytes. However, the exact mechanism of the suppression of ROI production of oyster hemocytes by *P. marinus* meronts is not known at this time and needs further examination.

Acknowledgements—This study was supported by a grant (No. NA16FL0402-01) from NOAA through the oyster disease program. The initial subcultures in our laboratory were generously provided by Dr. Frank Perkins. We thank Drs. Robert Hale, Peter Van Veld, Frank Perkins, Richard Lee, and the anonymous reviewers for critical review and helpful comments. Contribution #1927 from the School of Marine Science, Virginia Institute of Marine Science, College of William & Mary.

References

1. Andrews, J. D. Epizootiology of the disease caused by the oyster pathogen *Perkinsus marinus* and its effect on the oyster industry. Spec. Publ. Am. Fish. Soc. 18:47–63; 1988.
2. Burreson, E. M. Status of the major oyster diseases in Virginia—1989: A summary of the annual monitoring program: Marine Science Report 90-1, Virginia Institute of Marine Science, Gloucester Point; 1990.
3. Paynter, K. T.; Burreson, E. M. Effects of

Perkinsus marinus infection in the eastern oyster, *Crassostrea virginica*. II. Disease development and impact on the growth rate at different salinities. J. Shellfish Res. 10:425–431; 1991.
4. Chu, F. L. E.; La Peyre, J. F. *Perkinsus marinus* susceptibility and defense-related activities in eastern oysters, *Crassostrea virginica:* Temperature effects. Dis. Aquat. Org. 16:223–234; 1993.
5. Chu, F. L. E.; La Peyre, J. F.; Burreson, C. S. *Perkinsus marinus* infection and potential defense-related activities in eastern oysters, *Crassostrea virginica:* Salinity effects. J. Invert. Pathol. 62:226–232; 1993.
6. Allen, R. C.; Stjernholm, R. L.; Steele, R. H. Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leucocytes and its participation in bactericidal activity. Biochem. Biophys. Res. Commun. 47:679–684; 1972.
7. Thomas, E. L.; Lehrer, R. I.; Rest, R. F. Human neutrophil antimicrobial activity. Rev. Infect. Dis. 10:450–456; 1988.
8. Takeshige, K.; Minakami, S. Early events and stimulants triggering oxidative metabolism in neutrophils. In: Van Dyke, K.; and Castranova, V., Eds. Cellular chemiluminescence, Vol. 1. Boca Raton, FL: CRC Press; 1987:113–129.
9. Jones, H. P.; Ghali, G.; Petrone, M. F.; McCord, J. M. Calmodulin-dependent stimulation of the NADPH oxidase of human neutrophils. Biochimica et Biophysica Acta 741:152–156; 1982.
10. Chung, S.; Secombes, C. J. Analysis of events occurring within teleost macrophages during the respiratory burst. Comp. Biochem. Physiol. 89B(3):539–544; 1988.
11. Schlenk, D.; Martinez, P. G.; Livingstone, D. R. Studies on myeloperoxidase activity in the common mussel, *Mytilus edulis* L. Comp. Biochem. Physiol. 99C:63–68; 1991.
12. Bell, K. L.; Smith, V. J. In vitro superoxide production by hyaline cells of the shore crab *Carcinus maenas* (L.). Dev. Comp. Immunol. 17:211–219; 1993.
13. Ito, T.; Matsutani, T.; Mori, K.; Nomura, T. Phagocytosis and hydrogen peroxide production of the phagocytes of the sea urchin *Strongylocentrotus nudus*. Dev. Comp. Immunol. 16:287–294; 1992.
14. Nakamura, M.; Mori, K.; Inooka, S.; Nomura, T. In vitro production of hydrogen peroxide by the amoebocytes of the scallop, *Patinopecten yessoensis* (Jay). Dev. Comp. Immunol. 9: 407–417; 1985.
15. Dikkeboom, R.; Tijangel, J. M. G. H.; Mulder, E. C.; Van Der Knaap, W. P. W. Hemocytes of the pond snail *Lymnaea stagnalis* generate reactive forms of oxygen. J. Invertebr. Pathol. 49:321–331; 1987.
16. Dikkeboom, R.; Van Der Knaap, W. P. W.; Van Den Bovenkamp, W.; Tijangel, J. M. G. H.; Bayne, C. J. The production of toxic oxygen metabolites by hemocytes of different snail species. Dev. Comp. Immunol. 12:509–520; 1988.
17. Shozawa, A. A reducing factor produced by hemocytes of *Biomphalaria glabrata* and its role in the host defense. Dev. Comp. Immunol. 10:636; 1986.
18. Le Gall, G.; Bachère, E.; Mialhe, E. Chemiluminescence analysis of the activity of *Pecten maximus* hemocytes stimulated with zymosan and host-specific Rickettsiales-like organisms. Dis. Aquat. Org. 11:181–186; 1991.
19. Bachère, E.; Hervio, D.; Mialhe, E. Luminol-dependent chemiluminescence by hemocytes of two marine bivalves, *Ostrea edulis* and *Crassostrea gigas*. Dis. Aquat. Org. 11:173–180; 1991.
20. Larson, K. G.; Roberson, B. S.; Hetrick, F. M. Effect of environmental pollutants on the chemiluminescence of hemocytes from the American oyster *Crassostrea virginica*. Dis. Aquat. Org. 6:131–136; 1989.
21. Fisher, W. S.; Wishkovsky, A.; Chu, F. L. E. Effects of tributyltin on defense-related activities of oyster hemocytes. Arch. Environ. Contam. Toxicol. 19:354–360; 1990.
22. Anderson, R. S.; Oliver, L. M.; Jacobs, D. Immunotoxicity of cadmium for the eastern oyster (*Crassostrea virginica* [Gmelin, 1971]): Effects on hemocyte chemiluminescence. J. Shellfish Res. 11(1):31–35; 1992.
23. Adema, C. M.; Van Der Knaap, W. P. W.; Sminia, T. Molluscan hemocyte-mediated cytotoxicity: The role of reactive oxygen intermediates. Rev. Aquat. Sci. 4:201–223; 1991.
24. Horan, T. D.; English, D.; McPherson, T. A. Association of neutrophil chemiluminescence with microbicidal activity. Clin. Immunol. Immunopathol. 22:259–269; 1982.
25. Welch, W. D. Correlation between measurements of the luminol-dependent chemiluminescence response and bacterial susceptibility to phagocytosis. Infect Immun. 9:370–374; 1980.
26. Baboir, B. M.; Kipnes, R. S.; Curnutte, J. T. The production of leukocytes of superoxide, a potential bacterial agent. J. Clin. Invest. 52: 741–744; 1973.
27. Fisher, W. S. Environmental influence on bivalve hemocyte function. Spl. Publ. Am. Fish. Soc. 18:225–237; 1988.
28. Feng, S. Y. Cellular defense mechanisms of oysters and mussels. Spl. Publ. Am. Fish. Soc. 18:153–168; 1988.
29. Chu, F. L. E. Humoral defense factors in marine bivalves. Spl. Publ. Am. Fish. Soc. 18: 178–188; 1988.
30. Sminia, T.; Van Der Knaap, W. P. W. Cells and molecules in molluscan immunology. Dev. Comp. Immunol. 11:17–28; 1987.
31. La Peyre, J. F. Studies on the oyster pathogen *Perkinsus marinus* (Apicomplexa): Interactions with host defenses of *C. virginica* and *C. gigas*, and in vitro propagation. Ph.D. dissertation, The College of William and Mary, Williamsburg, VA, 1993:110–135.
32. Bushek, D.; Allen, S. K.; Alcox, K. A.; Gustafson, R.; Ford, S. E. Dose response of the eastern oyster, *Crassostrea virginica*, to cultured cells of *Perkinsus marinus*, the agent

of Dermo disease. J. Shellfish Res. 13:313; 1994.
33. Gauthier, J. D.; Vasta, G. R. Continuous in vitro culture of the eastern oyster parasite *Perkinsus marinus*. J. Invert. Pathol. 62:321–323; 1993.
34. Scott, A. L.; Klesius, P. H. Chemiluminescence: A novel analysis of phagocytosis in fish. Dev. Biol. Stand. 49:243–254; 1981.
35. Andersch, M. A.; Szczypinski, A. J. Use of *p*-nitrophenyl phosphate substrate in determination of serum acid phosphatase. Am. J. Clin. Pathol. 17:571; 1947.
36. Mauel, J. Mechanisms of survival of protozoan parasites in mononuclear phagocytes. Parasitology 88:579–592; 1984.
37. Moulder, J. W. Comparative biology of intracellular parasitism. Microbiol. Rev. 49:298–337; 1985.
38. Bogdan, C.; Rollinghoff, M.; Solbach, W. Evasion strategies of *Leishmania* parasites. Parasitol Today 6:183–187; 1990.
39. Hall, B. F.; Joiner, K. A. Strategies of obligate intracellular parasites for evading host defenses. Immunoparasitol. Today 1:A22–A27; 1991.
40. Russell, D. G.; Talamas–Rohana, P. *Leishmania* and the macrophage: A marriage of misconvenience. Immunol Today 10:328–333; 1989.
41. McNeely, T. B.; Turco, S. J. Inhibition of protein kinase C by the *Leishmania donovani* lipophosphoglycan. Biochem. Biophys. Res. Commun. 148:653–657; 1987.
42. Sminia, T.; Barendsen, L. A. A comparative and histochemical study on the blood cells of the fresh water snails *Lymnea stagnalis, Biomphalaria glabrata,* and *Bulinus truncatus*. J. Morphol. 165:31–39; 1980.
43. Cheng, T. C.; Rodrick, G. E. Lysosomal and other enzymes in the hemolymph of *Crassostrea virginica* and *Mercenaria mercenaria*. Comp. Biochem. Physiol. 52B:443–447; 1975.
44. Yoshino, T. P. Phospholipase C-like activity in phagocytic cells of the Asian clam *Corbicula fluminea,* and its possible role in cell-mediated cytolytic reactions. J. Invert. Pathol. 51:32–40; 1988.
45. Yoshino, T. P.; Lodes, M. J.; Rege, A. A.; Chappell, C. L. Proteinase activity in miracidia, transformation excretory–secretory products, and primary sporocysts of *Schistosoma mansoni*. J. Parasitol. 79(1):23–31; 1993.
46. Weiss, E.; Dobson, M. E.; Dasch, G. A. Biochemistry of rickettsiae: Recent advances. Acta Virol. 31:271–286; 1987.
47. Mkoji, G. M.; Smith, J. M.; Pritchard, R. K. Antioxidant systems in *Schistosoma mansoni:* Evidence for their role in protection of the adult worms against oxidant killing. Int. J. Parasitol. 18:667–673; 1988.
48. Remaley, A. T.; Kuhns, D. B.; Basford, R. E.; Glew, R. H.; Kaplan, S. S. Leishmanial phosphatase blocks neutrophil O^{2-} production. J. Biol. Chem. 259:11173–11175; 1984.
49. Hervio, D.; Chagot, D.; Godin, P.; Grizel, H.; Mialhe, E. Localization and characterization of acid phosphatase activity in *Bonamia ostreae* (Ascetospora), an intrahemocytic protozoan parasite of the flat oyster *Ostrea edulis* (Bivalvia). Dis. Aquat. Orgs. 12:67–70; 1991.