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Acidification of the Phagosome in *Crassostrea virginica* Hemocytes Following Engulfment of Zymosan

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Abstract. Phagocytic hemocytes are responsible for engulfing and internally degrading foreign organisms within the hemolymph and tissue of the eastern oyster, *Crassostrea virginica*. Since rapid acidification of the phagosome lumen is typically essential for activation of hydrolytic and reactive oxygen intermediate (ROI) producing enzymes in vertebrate cells, we measured phagosomal pH in oyster hemocytes by using the emission fluorescence of two fluorescent probes, rhodamine and Oregon Green 488 (OG 488), conjugated to zymosan to determine whether oyster hemocyte phagosomes become acidified after phagocytosis of zymosan. The average pH of 1079 phagosomes within 277 hemocytes 1 h after phagocytosis of zymosan was 3.9 ± 0.03. Observations of 141 hemocytes with internalized zymosan by light microscopy revealed that, over a 60-min time period, 51% of highly granular hemocytes became partially granular, and 29% became agranular. In addition, 83% of partially granular hemocytes containing zymosan at time = 0 became agranular within 60 min. A comparison revealed that the phagosomes of agranular hemocytes were much more acidic (pH 3.1 ± 0.02) than those of highly granular hemocytes (4.9 ± 0.02; $P < 0.05$). These values are significantly lower than most reported in the literature for blood cells from metazoan organisms.

Introduction

Phagocytic hemocytes are the primary cells involved in the oyster's internal defense response against invading organisms (Alvarez *et al.*, 1989; McCormick-Ray and Howard, 1991). Oyster hemocytes morphologically resemble vertebrate monocytes and macrophages (Anderson, 1981; Adema *et al.*, 1991) and, like these immune cells, have the ability to recognize, engulf, and internally degrade a variety of particles within the hemolymph and tissue (Foley and Cheng, 1975). The phagocytic process begins with recognition of a foreign particle and its engulfment into a phagosome. Once sequestered within the phagosome, the particle is subjected to various hydrolytic enzymes and reactive oxygen intermediates (ROIs), which aid in destroying the particle (Adema *et al.*, 1991; Anderson *et al.*, 1995). Many of the enzymes contained within the hemocytes of bivalves, including lysozyme (Rodrick and Cheng, 1974), β-glucuronidase (Cheng and Rodrick, 1975), and myeloperoxidase (MPO) (Wojcik and Paynter, 1995), have acidic pH optima ranging from pH 4.5–5.5. In addition, phagocytosis of foreign organisms by vertebrate macrophages and polymorphonuclear leukocytes (PMNs), as well as by hemocytes of *Mytilus edulis*, is accompanied by rapid acidification of the phagosome lumen (Jensen and Bainton, 1973; Rathman *et al.*, 1996; Kroschinski and Renwrantz, 1988). This suggests that, as in vertebrate phagocytes, phagosomal acidification may be required for activation of hydrolytic and ROI-producing enzymes in bivalve hemocytes, and may therefore be essential for the hemocyte antimicrobial defense response in molluscs.

Over the past few decades, the protozoan parasite *Perkinsus marinus* has caused widespread oyster mortalities in the Atlantic coast region. Although oyster hemocytes readily engulf *P. marinus*, it appears that the parasite may be able to survive within the hemocyte (Chu and La Peyre, 1993). In fact, many microorganisms can escape intracellular destruction by blocking phagosome-lysosome fusion (Jones and Hirsch, 1972; Horwitz, 1983; Mauel, 1984) or phagosomal acidification (Horwitz and Maxfield, 1984; Black *et al.*, 1986; Sibley *et al.*, 1985; Crowle *et al.*, 1991), or by adapting to the acidic environment of the phagosome

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(Antoine *et al.*, 1990; Maurin *et al.*, 1992; Rathman *et al.*, 1996). Characterization of the oyster's normal defense response to foreign organisms is essential to understanding how oyster hemocytes respond to parasites such as *P. marinus*. Therefore, we measured phagosomal pH from hemocytes of *C. virginica* following phagocytosis of a noninfectious particle, zymosan, to characterize hemocyte phagosomal acidification in response to a nonpathogenic particle.

Measurement of pH with internalized zymosan labeled with pH-sensitive fluorescent dyes indicates that hemocyte phagosomes become highly acidified after phagocytosis of zymosan. Individual hemocytes containing zymosan that were continuously monitored over time underwent a morphological transition from highly granular to partially granular or agranular in appearance. A comparison of the two distinct morphologies showed that the phagosomes of the agranular hemocytes were much more acidic than those of the highly granular hemocytes.

Materials and Methods

Animals

Specimens of *Crassostrea virginica* were obtained from Horn Point Laboratory (Cambridge, Maryland). Oysters were maintained in aerated aquaria containing artificial seawater (Instant Ocean) at room temperature and at the salinity of the site from which they were collected, typically 12–16 ppt. Oysters were not fed during their captivity and were kept in the aquaria for at least 5 days before being used in any experiment.

Isolation of hemocytes

Hemolymph was collected from oysters by notching the valve, draining the mantle fluid, and withdrawing hemolymph from the adductor muscle sinus using a 10-ml syringe equipped with a 22-gauge, 1.5-in. needle. Hemolymph from two or three oysters was expelled slowly into a glass test tube and kept on ice to prevent cell clumping. Pooled hemolymph (1–2 ml) was layered on the cover slip of a glass-bottomed microwell (MatTek Corporation, Ashland, Massachusetts) for 15 min, during which time the hemocytes adhered to the cover slip. The monolayer was rinsed and covered with 0.2-μm filtered artificial seawater (FAS 30 ppt; Instant Ocean) buffered to pH 7.0 with 40 m*M* Hepes (Buffer 1).

Quantification of morphological transitions in granular hemocytes

Oyster hemocytes include two types of cells, granulocytes and hyalinocytes, which were classified on the basis of electron microscope studies (Cheng, 1996). Granulocytes are the most phagocytic hemocytes and contain many acidophilic or basophilic granules, whereas hyalinocytes contain few or no granules (Foley and Cheng, 1972; Cheng, 1996; Fisher, 1985). Because our study was conducted at the light microscope level, hemocytes were classified according to the overall granularity of the cytoplasm, as identified by their unstained appearance by light microscopy (600×). Hemocytes in which 90% or more of the cytoplasm was filled with highly refractile granules were termed "highly granular," and those that were 30%–89% granular were considered "partially granular." Hemocytes with 30% or less granular material were termed "agranular."

Zymosan, a preparation of *Saccharomyces cerevisiae* cell wall that is readily engulfed by oyster hemocytes (Austin and Paynter, 1995; Anderson *et al.*, 1995), was prepared by boiling (10 mg/100 ml distilled water) for 30 min. The solution was centrifuged (400 × *g*) for 5 min, washed with Buffer 1 (pH 7.0) three times, and resuspended in Buffer 1 (pH 7.0) at a final concentration of 10 mg/ml. Ten microliters of the zymosan solution was then layered over the hemocytes and incubated for 20 min to allow maximal uptake of zymosan (Anderson *et al.*, 1995). Hemocyte monolayers were washed after the 20-min incubation, covered with Buffer 1 (pH 7.0), and incubated an additional 10 min to allow time for any adhering zymosan to be fully engulfed. A total of 30 min after the addition of zymosan (time = 0), a central field of 4–8 hemocytes and 3–4 adjacent fields of cells per dish were visually selected by light microscopy (Nikon 60×, N.A. 1.4 objective) and monitored for an additional hour. All incubations and monitoring of hemocytes were performed at 25°C to promote rapid spreading and initial phagocytosis of zymosan (Fisher, 1985) and to allow comparison of data to other studies (Anderson *et al.*, 1992, 1995; Austin and Paynter, 1995). At the beginning of the time course, the central and adjacent fields of cells were noted and hemocytes with and without engulfed zymosan were identified and numbered. Numbered hemocytes were then scored as highly granular, partially granular, or agranular on the basis of their morphology. Because hemocytes are mobile, the cells were continuously monitored by noting the specific morphology of each cell and frequently returning to adjacent fields of cells to ensure that each hemocyte being monitored was correctly identified throughout the time course. Although many of these hemocytes became progressively agranular, the cells could still be easily identified according to their overall size and shape. The hemocytes were rescored at 30 and 60 min after time 0.

Three replicate experiments were performed. During experiment one, four monolayers containing a total of 65 hemocytes within selected fields were monitored. In experiment 2, two monolayers were prepared and 39 hemocytes monitored. Experiment 3 consisted of 4 monolayers and 37 hemocytes. The total number of hemocytes monitored was 141.

Preliminary phagosomal pH experiments

Preliminary experiments using the ratiometric fluorescence method of pH quantification, which uses the ratio of the emission intensities of a pH-sensitive dye (fluorescein) and a pH-insensitive dye (rhodamine) to quantify pH (Dunn *et al.*, 1994), indicated that agranular hemocyte phagosomes became acidified beyond the sensitivity range of fluorescein, which is strongly quenched below pH 5.0 (Haugland, 1996). This ratiometric method was modified by substituting Oregon Green 488 (OG 488), a fluorophore that is sensitive between pH 6.0 and 2.0, for fluorescein. Dual labeling of zymosan with rhodamine and OG 488 has been used to measure phagosomal pH in macrophages (Vergne *et al.*, 1998).

Fluorescent labeling of zymosan

Zymosan was prepared for conjugation with fluorescent probes by boiling (10 mg/100 ml distilled water) for 30 min. The solution was centrifuged (400 × *g*) for 5 min, washed with fresh 0.1 *M* $NaHCO_3$ buffer (Buffer 2), pH 8.3, three times, and resuspended in Buffer 2 at a final concentration of 10 mg/ml.

The active succinimidyl ester forms of carboxytetramethylrhodamine (TMR) and OG 488 were dissolved in anhydrous *N,N*-dimethylformamide (DMF) (3 mg/ml) and added to the zymosan suspension. The final reaction mixture (9.09 mg/ml zymosan, 0.27 mg/ml TMR, 0.27 mg/ml OG 488) was stirred at room temperature for 2 h. Labeled zymosan was removed from the mixture by centrifugation (15,000 × *g*). The pellet was resuspended in Buffer 2, centrifuged for 30 s (15,000 × *g*) and washed (Buffer 2) three times. The labeled zymosan was resuspended in Buffer 2 (10 mg/ml) and stored in 1-ml aliquots in the dark (−4°C) until use.

Calibration images

Quantification of pH was obtained using a ratio of the emission intensities of rhodamine, which is pH insensitive, and OG 488, which is pH sensitive. Images of fluorescent zymosan were collected with a microscope (Nikon 60×, N.A. 1.4 objective) equipped with a krypton-argon laser scanning confocal attachment (BioRadMRC 1024). Fluorescent zymosan was excited (488 nm) and OG 488 emission (green light) was collected using a 522/32 nm band pass filter. Rhodamine emission (red light) was collected using a 598/40 nm band pass filter. Laser power, photomultiplier gain, confocal aperture, and background levels were adjusted manually.

Extracellular calibrations. Calibration images were obtained by centrifuging labeled zymosan (15,000 × *g*) for 30 s, and resuspending zymosan (10 mg/ml) in Buffer 1 titrated to pHs of 6.0, 5.0, 4.0, 3.0, and 2.0. Aliquots of labeled zymosan at each pH were layered on the coverslip of a microwell and three fields of zymosan at each pH were selected, scanned with a krypton-argon laser (488 nm), and images collected digitally.

Intracellular calibrations. To determine if internalization of zymosan within hemocytes had any effect on the ratio of emission intensities of the two fluorescent dyes, intracellular calibrations were performed. Hemocyte monolayers were prepared as described previously. The microwells were filled with Buffer 1 (pH 7.0) so that the bottom of each microwell was fully covered. Labeled zymosan was centrifuged (15,000 × *g*) for 30 s and resuspended (10 mg/ml) in Buffer 1. Ten microliters of this solution was layered just above the hemocyte monolayer. The monolayers were placed in the dark and incubated for 30 min. After incubation, the monolayers were washed thoroughly with Buffer 1 (pH 7.0) and then covered with Buffer 1 (pH 7.0) containing 4% formaldehyde for 15 min. The monolayers were washed three times with Buffer 1 (pH 7.0) and placed in the dark. Monensin, a Na^+ ionophore used to neutralize pH gradients (Horwitz and Maxfield, 1984), was dissolved in 95% ethanol (1 m*M*) and added to Buffer 1 titrated to pH 6.0, 5.0, 4.0, 3.0, and 2.0 for a final concentration of 50 μM. Hemocyte monolayers were washed three times in the appropriate pH buffer and then immersed in Buffer 1 (pH 6.0, 5.0, 4.0, 3.0, or 2.0) containing 50 μM monensin. For each pH, 10 hemocytes with internalized zymosan were selected visually (600×) and scanned with the confocal laser as described above.

Measurement of phagosomal pH

Hemocyte monolayers were prepared and incubated with zymosan as described for the intracellular calibrations. However, after the 30-min incubation period, the monolayers were washed to remove any extracellular zymosan, covered again with Buffer 1 (pH 7.0), and incubated for another 30 min to ensure that any zymosan adhering to the hemocyte cell surface was fully engulfed. After the 60-min incubation period, hemocytes with internalized zymosan that appeared highly granular, partially granular, or agranular were visually selected and scanned with the confocal laser as described previously. Within a 15-min time period, about 25 hemocytes containing internalized zymosan were imaged per microwell. Four separate experiments (experiment 1–4) were performed on different days. In experiment 1, 3 monolayers were prepared and 69 hemocytes containing a total of 300 zymosan particles were selected and scanned. In experiment 2, 120 hemocytes with 430 zymosan particles were scanned from four monolayers. In experiments 3 and 4, two monolayers were prepared and 44 hemocytes with 202 zymosan particles (experiment 3) and 44 hemocytes with 147 zymosan particles (experiment 4) were scanned. Overall, 277 hemocytes containing 1079 engulfed particles of zymosan were scanned. Of these he-

mocytes, 83 were agranular (containing a total of 364 zymosan) and 83 were highly granular (containing 297 zymosan). Hemocytes containing unlabeled zymosan were also scanned to ensure that the fluorescent emission detected was not due to the autofluorescence of hemocytes or zymosan.

Image analysis

All images were processed and analyzed using the NIH-Image v. 1.61 software package (Wayne Rasband, National Institutes of Health). Rhodamine and OG 488 emissions were analyzed separately. First, the background fluorescence was subtracted from each image. The image was then duplicated and a threshold was applied to include zymosan with intensities of at least 2 on a scale of 256 grays (where 255 is the most intense, and 0 corresponds with no emission intensity). Each image was binarized, and the two resulting images were multiplied to create a mask image of pixels shared by both rhodamine and OG 488 emissions. The mask image was multiplied by each original background-corrected image to eliminate any unshared pixels. The final rhodamine image was divided by the final OG 488 image to obtain an image that was a ratio of the rhodamine to OG 488 emission intensities. Each zymosan particle was then analyzed manually by selecting an area and measuring the mean rhodamine/OG 488 fluorescence intensity for that set of pixels.

Data analysis

All data were analyzed using a statistical software package (StatView, Abacus Concepts, Berkeley, California). The ratio of rhodamine to OG 488 fluorescence for measurements of intracellular phagosomal pH were converted to pH values using a second- or third-order polynomial regression equation obtained from the calibration images collected for each experiment. Differences in mean phagosomal pH over time and between agranular and granular hemocytes were analyzed with a one factor factorial ANOVA. The percentages of granular and agranular hemocytes at times 0, 30, and 60 min were analyzed with a one factor ANOVA to determine whether the percentages of hemocytes types were statistically different between time points. Data groups were considered significantly different if $P < 0.05$.

Materials

Zymosan, DMF, and monensin were obtained from Sigma (St. Louis, Missouri). OG 488, Cl-Nerf, fluorescein, and TMR were purchased from Molecular Probes (Eugene, Oregon).

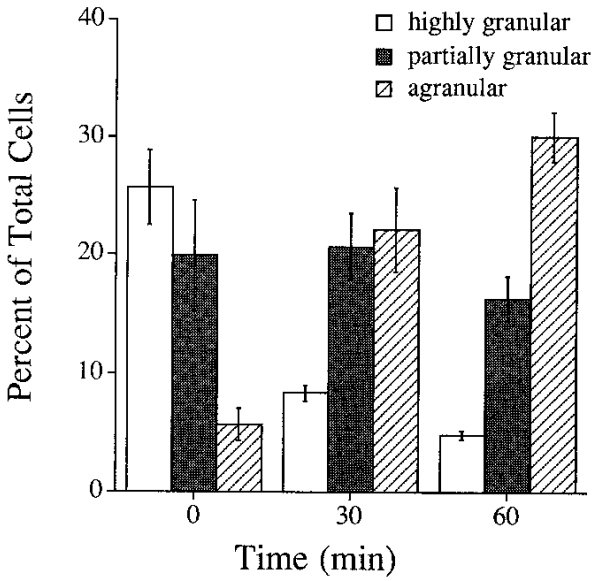

Figure 1. Percentage of 72 highly granular, partially granular, and agranular hemocytes containing zymosan at each time interval following a 30-min incubation period with zymosan (see text for specific conditions). The 69 hemocytes that did not contain zymosan are not shown because no change in morphology occurred. Error bars represent the standard error of the means.

Results

Morphological transition of hemocytes over time

The results of three experiments in which a total of 141 hemocytes were monitored continuously for 1 h indicate that granular hemocytes containing internalized zymosan undergo a morphological transition over time. A comparison of the total population of highly granular, partially granular, and agranular cells containing zymosan at times 0, 30, and 60 min indicated that the percentage of highly granular cells decreased over time, while the percentage of agranular cells increased (Fig. 1). In addition, because individual hemocytes were monitored continuously, the total number of zymosan-containing hemocytes that changed in morphology between each time point could be quantified (Table I). Most of the highly granular hemocytes with internalized zymosan identified at time = 0 became partially granular over a 30-min period, and less frequently they changed directly from highly granular to agranular within this time (Table I). Of those hemocytes at time = 0 that were partially granular, 83% became agranular within 1 h (Table I). Zymosan-containing hemocytes that remained granular throughout the time course (Table I) typically had only one or two engulfed zymosan particles, whereas those that became increasingly agranular over time typically had three or more internalized particles. In addition, 83% of the cells whose morphology remained the same throughout the

Table I

Number of cells of specific hemocyte types—with (z+) and without (z−) engulfed particles of zymosan—that exhibited a change in morphology over a 60-minute time course, compared with hemocytes (z+ and z−) that did not change in morphology

	Change in morphology							
	Highly granular to partially granular		Partially granular to agranular		Highly granular to agranular		No Change in morphology	
Time period (minutes)	z+	z−	z+	z−	z+	z−	z+	z−
0 to 30	17	0	17	0	6	0	7	57
30 to 60	5	0	7	0	0	0	5	0
Total	22	0	24	0	6	0	20	69

Hemocytes were identified as highly granular, partially granular or agranular after a 30-min incubation with zymosan (time 0; see text for specific conditions) and were continually observed for an additional 60 minutes. Morphologies of a total of 141 hemocytes at 0, 30 and 60 minutes were recorded.

time course contained no zymosan, and the hemocytes—with and without zymosan—that were agranular at time = 0 remained agranular throughout the time course (Table I).

pH calibration curves

Extracellular and intracellular calibration data collected before each experiment fit a second- or third-order polynomial regression equation with an adjusted R^2 of at least 0.94 ($P < 0.0001$; Fig. 2). Intracellular calibrations in which hemocytes with internalized zymosan were treated with monensin and equalized with buffer resulted in rhodamine/OG 488 emission ratios that were not statistically different from fluorescent emission ratios of extracellular zymosan at each pH. Because fluorescence emission of OG 488 was recoverable after addition of monensin to hemocyte monolayers, the emission intensities of the dyes were not altered by factors other than pH.

Phagosomal pH of random hemocytes

After the 30-min exposure to zymosan, hemocytes typically contained from 1 to 10 internalized zymosan particles. The results of four experiments indicate that the average pH of a total of 1079 phagosomes within 277 hemocytes at 60 min was 3.9 ± 0.03, with the distribution of pH values ranging from 5.9 to 2.4.

Phagosomal pH of granular and agranular hemocytes

The pH values of 297 phagosomes within 83 granular hemocytes from four experiments ranged from pH 3.8 to 5.9, with a mean pH of 4.9 ± 0.02 (Fig. 3). In contrast, the mean pH of 364 phagosomes within 83 agranular hemocytes was much lower (pH 3.1 ± 0.02; $P < 0.05$). The pH of phagosomes in agranular hemocytes ranged from 2.4 to 4.0 (Fig. 3).

Discussion

The average pH of eastern oyster hemocyte phagosomes after engulfment of zymosan falls below the ranges reported in the literature for most phagocytic cells (Table II). Phagosomal acidification, which is probably necessary for activation of digestive enzymes, varies depending on the or-

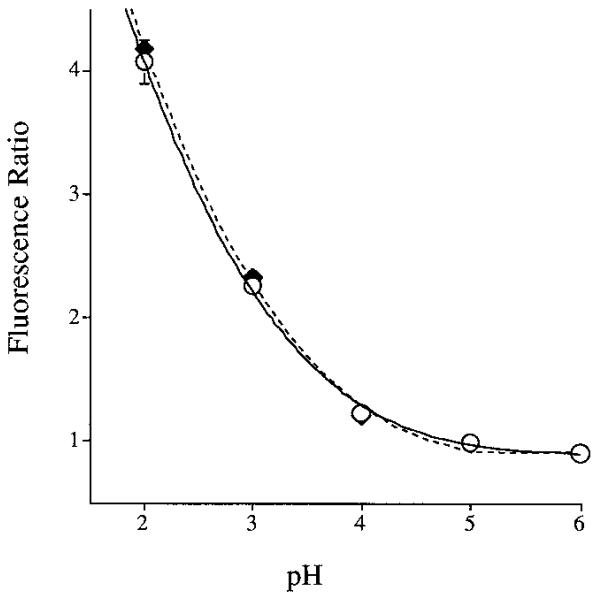

Figure 2. Example of a calibration curve for determination of pH values from the ratio of rhodamine to OG 488 emission intensities. Calibration curves were generated from extracellular zymosan placed in buffers of different pH values (- ○ -) or from zymosan engulfed within hemocytes that were fixed with 4% formaldehyde and incubated in buffers of different pH values containing 50 μM monensin to dissipate the pH gradient (- - ◆ - -). Ratio data were fitted to a second- or third-order polynomial regression equation that was then used to determine the pH of the zymosan engulfed within the hemocytes. Error bars indicate one standard deviation of the mean.

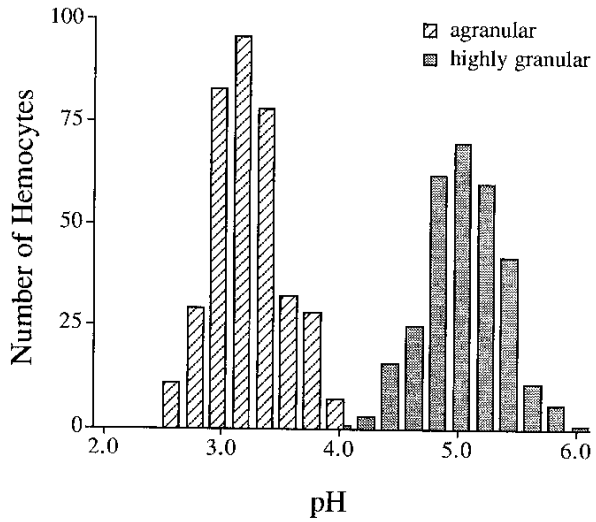

Figure 3. Distribution of pH associated with zymosan engulfed by granular and agranular hemocytes after a 30-min exposure to zymosan and an additional 30-min incubation. Hemocytes were classified as granular or agranular on the basis of the overall granularity of the cytoplasm as observed by light microscopy, and the pH of engulfed fluorescent zymosan was calculated from the ratio of the emission intensities of rhodamine and OG 488. The mean pH was 4.9 ± 0.02 for 297 phagosomes within 83 granular hemocytes, but much lower (mean pH 3.1 ± 0.02; $P < 0.05$) for 364 phagosomes within 83 agranular hemocytes.

ganism and cell type. For instance, the lowest phagosomal pH value reported in mouse macrophages after phagocytosis of yeast was 5.0 (Geisow *et al.*, 1981), and hemocyte phagosomes of *Mytilus edulis* reached a low pH of 4.5 ± 0.5 (Kroschinski and Renwrantz, 1988). In contrast, digestive vacuoles of *Paramecium* become acidified to pH 3.0 after acidosomes bind with the digestive vacuole (Fok and Allen, 1990). In most cells, acidification of the phagosome lumen may occur in as little as 7–15 min after engulfment of foreign material (Jensen and Bainton, 1973; Geisow *et al.*, 1981; Bassoe and Bjerknes, 1985), and the phagosome in some cell types can remain acidified for 5 h or more after phagocytosis (Horwitz and Maxfield, 1984; Rathman *et al.*, 1996).

Like the digestive vacuoles of *Paramecium*, the hemocyte phagosomes of *C. virginica* become highly acidified. However, the degree of acidification varies, and this variation appears to be related to the final morphology of the hemocyte. Most of the individual hemocytes monitored over time underwent a morphological transition from highly granular to increasingly agranular in appearance after engulfment of zymosan, and measurements of phagosomal pH revealed that hemocytes remaining highly granular were not as acidic as those of the agranular form. In fact, preliminary experiments that used fluorescein as the pH-sensitive dye were unsuccessful due to the very low pH of the agranular hemocyte phagosome.

The granular hemocytes of bivalves contain many cytoplasmic granules and morphologically resemble vertebrate immune cells such as macrophages and PMNs (Cheng, 1996; Adema *et al.*, 1991). Like their vertebrate counterparts, hemocytes may degranulate after engulfing foreign particles (Cheng, 1996). In vertebrate PMNs, degranulation of the cytoplasm only takes place after engulfment of a foreign particle (Hirsch, 1962), and typically occurs within 30 min after phagocytosis (Hirsch and Cohn, 1960). Similarly, the transition in oyster hemocyte morphology typically occurred within 0–30 min after engulfment of zymosan, and granular hemocytes without engulfed particles remained granular throughout the time course. This suggests that granular hemocytes containing zymosan became more agranular in appearance over time as the result of increasing phagosome-lysosome fusion and subsequent degranulation of the hemocyte cytoplasm. This also suggests that the highly acidic pH of the agranular hemocyte phagosome may be the result of increased lysosomal fusion. Although *Paramecium* phagosomes subsequently become alkaline after lysosomal fusion (Fok and Allen, 1990), degranulation of mouse macrophages is associated with a gradual reduction in phagosomal pH (Geisow *et al.*, 1981). *Amoeba proteus* phagosomes also become further acidified as lysosomal fusion occurs (McNeil *et al.*, 1983).

The corresponding decrease in pH from the granular to agranular morphological state suggests the hemocyte is attempting to digest the engulfed zymosan. A similar rapid

Table II

Typical phagosomal pH values and method of measurement for a variety of phagocytic cells

Organism	Cell type	Target particle	Minimum pH of phagosome	Method	Source
rat	PMN*	yeast	3.5–4.5	indicator dyes	Jensen & Bainton, 1973
mouse	macrophage	yeast	5.0	fluorescein	Geisow *et al.*, 1981
mouse	macrophage	latex beads	4.0–5.0	DM-Nerf	Rathman *et al.*, 1996
Mytilus edulis	hemocyte	yeast	4.5 ± 0.5	indicator dyes	Kroschinski & Renwrantz, 1988
Paramecium		yeast	3.0	fluorescein	Fok & Allen, 1990

* PMN—polymorphonuclear leukocyte.

decrease in pH occurs in *Paramecium,* in which the phagosomal pH decreases from 7.0 to 3.0 after the digestive vacuole containing the phagocytized material binds with acidosomes, initiating the process of prey killing and protein denaturation (Fok and Allen, 1990). Electron micrographs of degranulated bivalve hemocytes show that digestive lamellae form around partially degraded foreign material engulfed within the phagosome, and numerous glycogen granules appear in the cytoplasm (Cheng and Foley, 1975). On the basis of these observations, Cheng and Foley (1975) proposed that degranulated hemocytes were in the process of intracellular digestion of engulfed materials.

The lowest measured pH of oyster hemocyte phagosomes (pH 2.4) is much more acidic than the lowest pH value estimated from hemocyte food vacuoles of *Mytilus edulis* (Table II). However, it is important to note that these researchers did not report any change in the granularity of the hemocytes (Kroschinski and Renwrantz, 1988). In addition, the average pH of the agranular form of hemocytes was much lower than the pH optima of digestive enzymes contained within molluscan hemocytes (4.5–5.5). This suggests that these enzymes may be inactivated or that perhaps other enzymes with more acidic pH optima become active. Alternatively, it is possible that a subsequent alkalinization of the phagosome occurs, as in *Paramecium* and *Chaos carolinensis* (Fok and Allen, 1990; Heiple and Taylor, 1982). However, we have observed that agranular hemocyte phagosomes remained acidified for up to 4 h after phagocytosis (data not shown).

As in vertebrate immune defense cells, phagocytosis of foreign organisms by oyster hemocytes causes a biochemical cascade resulting in the production of ROIs such as superoxide anion and hypochlorous acid (HOCL) which contribute to oxidative killing of phagocytized foreign microorganisms (Adema *et al.,* 1991; Anderson *et al.,* 1992). The production of ROIs by oyster hemocytes reaches a peak 10 to 15 min after introduction of zymosan to hemocytes, and then gradually declines over a period of 120 min (Austin and Paynter, 1995). Myeloperoxidase (MPO), the enzyme responsible for the production of HOCL, was partially purified from oyster hemocytes and shown to have a pH optimum of 5.5 (Wojcik and Paynter, 1995). The phagosomal pH of granular hemocytes (mean pH 4.9 $\pm$ 0.02) is very close to the pH optimum of MPO. Thus, the production of ROIs by hemocytes just after stimulation may be the hemocyte's first line of defense against invading organisms. Over time, granular hemocytes become increasingly agranular in appearance as lysosomes fuse with the phagosome, resulting in a concomitant decrease in pH. The production of ROIs may also decline as the pH decreases below the optimal pH of the ROI-producing enzymes such as MPO. In fact, at pH values less than 4, only 40% of MPO would be active (Wojcik and Paynter, 1995).

In conclusion, highly and partially granular hemocytes are most often associated with internalized zymosan at the beginning of the time course. Corresponding with this early time point and morphological state is a mean pH similar to optimal pH values of hydrolytic and ROI-producing enzymes. Over a period of 60 minutes, however, the hemocyte becomes increasingly agranular as lysosomes apparently fuse with the phagosome, further reducing the pH within the phagosome. Although the pH within the phagosome of the highly granular hemocyte is comparable to that of most other phagocytic cells, the phagosomal pH of the agranular hemocyte is more typical of the digestive vacuole in *Paramecium,* which reaches a low pH of 3.0 (Fok and Allen, 1990), suggesting that phagosomal acidification is a vital component of the oyster hemocyte's defense response.

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