FLOW CYTOMETRIC MEASUREMENT OF HEMOCYTE VIABILITY AND PHAGOCYTIC ACTIVITY IN THE CLAM, *RUDITAPES PHILIPPINARUM*

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ABSTRACT The assessment of blood cell viability and defense abilities is a major concern in the study of pathological processes. In this work, we devised and validated flow cytometric assays to measure viability and phagocytic activity of hemocytes from the clam *Ruditapes philippinarum*, a species susceptible to the bacteria-caused Brown Ring Disease (BRD). Validated assays were subsequently used to measure hemocyte parameters following experimental contamination with BRD's etiologic agent *Vibrio tapetis*. Results show that clams that developed BRD symptoms had lower phagocytic rates and a higher percentage of dead hemocytes than those that did not. *In vitro* interactions between hemocytes and *V. tapetis* demonstrated that clam hemocytes are able to phagocytose formalin-fixed *V. tapetis*, but at lower rates than latex beads. Live *V. tapetis* were able to kill clam hemocytes *in vitro*. The *in vitro* assay also showed that phagocytosis increased with increasing temperature from 8° to 21°C. This work demonstrated the efficiency of flow cytometry for measuring molluscan blood cell activities during host/pathogen interactions and points the way for further experiments using this analytical tool.

KEY WORDS: bivalve, bacteria, phagocytosis, hemocyte viability, flow cytometry, *Ruditapes philippinarum*

INTRODUCTION

Changes in overall hemocyte activity have been observed in parasitized bivalves or those experimentally exposed to pathogens (Nottage & Birkbeck 1990; La Peyre et al. 1995; Anderson 1996; Garreis et al. 1996; Allam et al. 2000a, 2000b). Techniques used to investigate these changes generally have serious drawbacks. Microscopy is subjective and involves a considerable expenditure of time, especially when large numbers of samples need to be evaluated. Spectrophotometric methods are based on the measurement of activity in the whole sample and not on a cell-by-cell basis. Flow cytometry, however, has proven to be extremely useful in overcoming these problems. In the field of molluscan research, flow cytometry has been used to quantify phagocytosis (Alvarez et al. 1989; Brousseau et al. 2000; Allam et al. 2001; Fournier et al. 2001) and hemocyte viability (Ashton-Alcox & Ford 1998; Ashton-Alcox et al. 2000; Fournier et al. 2001).

In flow cytometry, particles are passed single-file through a laser beam. The light scattered by the particles indicates their size and internal complexity. Fluorescence, whether autofluorescence or from a fluorescent tag, is measured by specific detectors. Flow cytometric phagocytosis measurements usually involve the use of fluorescent particles that are detectable by the flow cytometer even after phagocytosis has taken place (Alvarez et al. 1989; Brousseau et al. 2000). Specific fluorescent dyes that indicate membrane integrity and permeability, intracellular redox potential, or enzymatic activity are available and can indicate cell viability using flow cytometry (Combrier et al. 1989).

Although molluscan hemocytes have been studied by flow cytometry before, validation of the results is rarely reported. In this work, we refined and verified by microscopy, two flow cytometry-based assays to measure the phagocytic activity and the viability of hemocytes in the clam *Ruditapes philippinarum*. These allowed us to investigate hemocyte activity in clams experimentally infected with *Vibrio tapetis*, the bacterial agent of Brown Ring Disease (BRD) in Europe (Paillard et al. 1994). We further studied the effect of *in vitro* contact between the pathogenic bacterium and hemocytes on hemocyte viability, and compared the uptake of *V. tapetis* with that of similarly sized fluorescent beads. We also investigated the effect of temperature on phagocytosis because temperature is strongly associated with the prevalence and intensity of BRD in nature and under experimental conditions (Paillard et al. 1994; Allam 1998).

MATERIALS AND METHODS

Experimental Animals

Ruditapes philippinarum were obtained from 2 locations: southern Puget Sound, Washington, USA (length = 39.4 ± 0.46 mm, mean ± SEM), and the Bay of Brest, Brittany, France (length = 37.7 ± 0.65 mm). Clams were shipped overnight to the laboratory where they were immediately placed in quarantine, aerated 35-L standing-water tanks (about 35 clams per tank at 13°C and 34 ppt), in which they were kept during the remainder of the study. Clams were fed daily throughout the experiments using a mixture of cultured algae. All the experiments reported here were conducted using clams from the USA, where BRD has never been reported, except for the infection experiment, which was done using French clams.

Clams affected with BRD were obtained by challenging them with *V. tapetis* as previously described (Allam et al. 2000a). Briefly, a 0.5 ml (5×10^7 bacteria) aliquot of a suspension made with exponentially growing *V. tapetis* (ATCC 4600, strain P16) was inoculated into the pallial cavity of each experimental clam. Control clams were inoculated with the same volume of sterile seawater. After 4 weeks of incubation under the conditions described above, hemolymph was collected and processed for phagocytosis and viability assays. The clams were then shucked and BRD development on shells was recorded (Paillard & Maes 1994; Allam et al. 2000a).

Hemolymph Sampling

Animals and hemolymph samples were kept on ice during processing to avoid hemocyte clumping. Hemolymph was withdrawn from the posterior adductor muscle as described by Auffret and

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Oubella (1995). Samples were immediately divided into aliquots and diluted in different solutions as described below.

Design and Validation of the Phagocytic Assay

In vitro Incubation of Hemocytes with Beads

Fluorescent latex beads, 2.02 μm in diameter (Fluoresbrite Calibration grade, Polysciences, USA), were dispersed by expelling them through a 26-gauge needle and diluting them with filtered seawater (FSW) to give a final concentration of 6×10^6 beads ml^{-1}. Two hundred microliters of this suspension were placed in each well of a 24-well microplate, which was centrifuged for 10 min at $200 \times g$ at room temperature (~21°C) to form a uniform monolayer of beads on the bottom of each well. Hemolymph samples were immediately diluted with ice-cold sterile seawater (SSW) to give a final concentration of 5 to 7×10^5 cells ml^{-1} and 200-μl aliquots of these suspensions were added to each well to give approximately a 1:10, cell:bead ratio. Following a 30-minute incubation in thermostatic chambers (Minifrige II®, Boekel Industries, Inc., Philadelphia, PA) adjusted to 21°C, conditions determined to be optimal during preliminary assays, the cytoskeleton-inhibitor Cytochalasin B (10 μg ml^{-1}, final concentration) was added to each experimental well to stop hemocyte activity. Attached cells were released by trypsinization (0.4% trypsin in 1% EDTA-saline solution for 10 min) followed by gentle sonication for one minute at room temperature (RT). Microscopic observation verified that this procedure detached cells from wells and also released non-ingested particles from the surfaces of hemocytes. Formalin was then added for a final concentration of 3% to fix the sample, which was transferred to a microfuge tube where it was held on ice until processed, within an hour, by flow cytometry. Because the flow cytometric profile for hemocytes from each clam was unique (see later), it was necessary to have a control (i.e., no phagocytosis) profile for each individual. Thus, a control well was established for each clam in which Cytochalasin B was added at the beginning of the incubation period to prevent phagocytosis. After the incubation, the control wells received the trypsin and formalin treatments as described above.

Flow Cytometry

Flow cytometry was performed on a Coulter EPICS C equipped with an argon laser and operated at a wavelength of 488 nm. Gains and photomultiplier high voltage settings were adjusted to include all cell and bead particles. Forward light scatter (FLS) and green fluorescence (GFL) list mode data were collected. A total of 10,000 particles were counted for each sample. The percent phagocytosis was calculated for each clam by bitmapping (electronic outlining) each of three particle types: (1) free beads; (2) non-bead-associated cells; and (3) bead-associated cells (Fig. 1). The bitmaps had been established previously by running beads alone, then non-bead-associated cells plus free beads, and then samples with phagocytosed beads. For each clam, the sum of counts in bitmaps 2 and 3 represented the total hemocytes in the flow cytometry sample. In each sample, the percent phagocytosis was computed as the ratio of bead-associated hemocytes to total hemocytes × 100. For each clam, the percent phagocytosis was calculated as the difference between the percent phagocytosis in the test wells and the percent phagocytosis in the control well. The percent phagocytosis was always below 0.5% in the control wells. For each sample, mean fluorescence intensity (channel number) was calculated within each bitmap.

Assay Validation

Epifluorescence microscopy was used to compare and correlate the percent phagocytosis results obtained from flow cytometry. Bead-associated and non-bead-associated hemocytes were counted using a Zeiss ICM 405 microscope equipped with a standard FITC filter set. A minimum of 250 cells was counted in each sample. This comparison included control samples with added Cytochalasin B. In addition, the reproducibility of the assay was tested by establishing duplicate experimental wells for each clam and comparing flow cytometric results for the replicates using correlation analysis. Finally, to determine the effect of storage on the percent phagocytosis measurement, samples were collected as described above, processed by flow cytometry, stored in glass tubes at 4°C, and re-analyzed after 3 and 7 days.
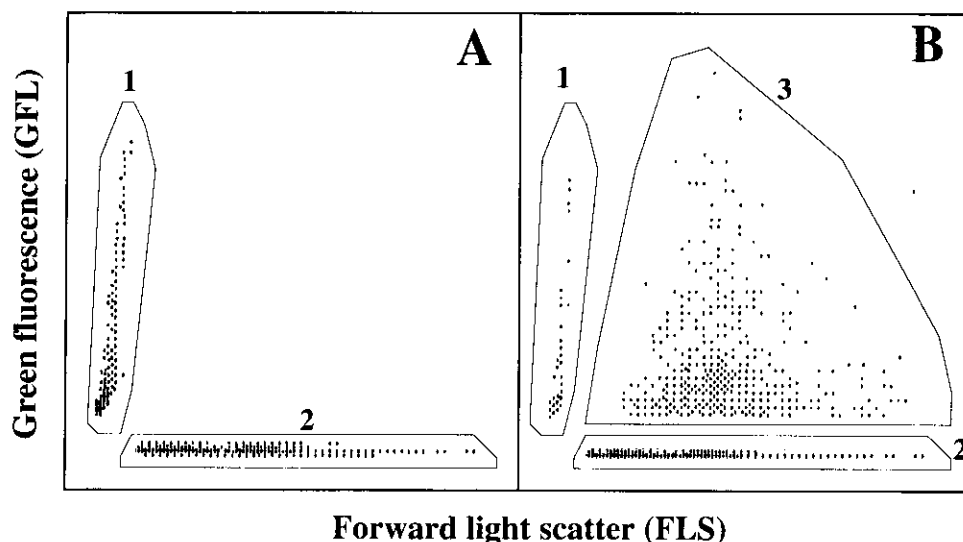

Figure 1. Flow cytometry bivariate plots showing the bitmaps used to calculate bead-associated and non-bead-associated cells in control (A) and test (B) mixtures. Bitmap 1: beads alone, Bitmap 2: non-bead-associated cells, Bitmap 3: bead-associated cells.

Design and Validation of the Viability Assay

In vitro Incubation of Hemocytes with the Fluorescent Vital Stain

The percentage of dead cells was assessed using the fluorescent nucleic acid stain ethidium homodimer-1 (EHD) previously used by Ashton-Alcox and Ford (1998). EHD binds to nucleic acids by intercalation. It does not permeate cells with intact membranes; thus only dead cells, or those with damaged membranes, become fluorescent. Hemolymph samples were diluted in cold Alsever's solution (1:10, v:v) immediately after collection. Then, 2 μM EHD (Molecular Probes, Eugene, Oregon, USA), dissolved in DMSO according to manufacturer's directions, was added. The mixture was incubated at room temperature for 30 minutes. An unstained control sample was made for each clam.

Flow Cytometry

Flow cytometry was used to collect light scatter parameters and log red fluorescence (LRFL) signals for at least 5000 cells. The percentage of dead cells was determined by setting a cursor at the upper limit of the LRFL signal for the unstained control, which was used as the "zero" channel for the stained cells in the parallel treated sample (Fig. 2). The percentage of dead cells was calculated as the ratio of cells above the "zero" channel to total hemocytes × 100.

Assay Validation

Fluorescence microscopy was used to compare and correlate the hemocyte viability results obtained from flow cytometry. Fluorescent and non-fluorescent hemocytes were counted using an epifluorescence microscope as described above. A minimum of 200 cells was counted in each sample. The reliability of the assay was also tested by using hemocytes that had been killed by immersion in boiling water for 5 min. Hemocyte mortality was confirmed microscopically, after EHD uptake, to be 100%, whereas untreated cells were more than 96% viable. Five mixtures of untreated and

heat-killed hemocytes were made using 0%, 25%, 50%, 75%, and 100% heat-killed hemocytes. The percentage of viable and non-viable hemocytes was measured flow cytometrically as described above.

Effect of Temperature on Phagocytosis

Because BRD development is partially controlled by temperature, the phagocytosis assay was used to quantify the effect of temperature on phagocytosis by R. philippinarum hemocytes in vitro. Hemolymph was withdrawn from clams and immediately diluted with ice-cold SSW to give a final concentration of about 5 to 7 × 10^5 cells ml^{-1}. For each clam, 6 test wells and one control well of hemocytes and beads were established. Two replicates were incubated at 8°C, two at 13°C, and two at 21°C in the Mini-frige II$^®$ temperature chambers. These temperatures were selected because they are associated with the development of significantly different BRD prevalences and intensities in experimentally challenged clams (Allam 1998). The single control well was incubated at 21°C since preliminary studies showed no evidence of phagocytosis in controls at any tested temperature. All samples were processed for flow cytometry as described above.

In vitro Interactions Between Hemocytes and V. tapetis

The first experiment was devised to study the uptake of fluorescently labeled V. tapetis by hemocytes. Exponentially growing V. tapetis, cultured on marine agar were suspended in phosphate-buffered saline (PBS) to obtain about 10^9 cfu ml^{-1}. The bacteria were then fixed in 2% formalin, washed 3 times with PBS, and resuspended in 1 ml PBS (pH 7.4) containing 1 mg FITC (Sigma). The mixture was incubated for 30 min at RT, washed twice in PBS, and finally resuspended in sterile seawater. Labeled bacteria were then placed in each well of a 24-well microplate, centrifuged to form a uniform layer on the bottom of each well (10 min, 500 × g, 21°C) and used for measuring the phagocytic activity of hemocytes as described above. Six wells were established for each
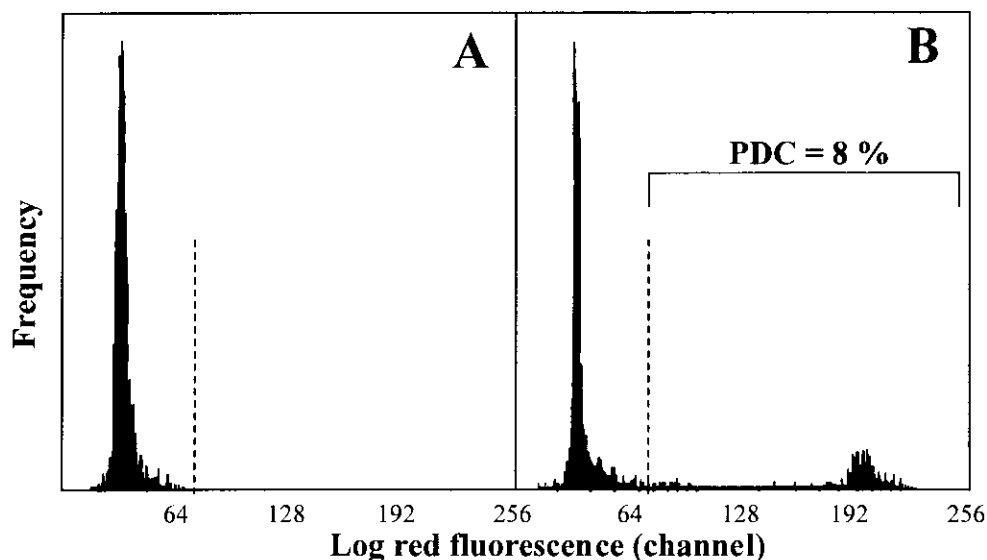

Figure 2. Distribution of log red fluorescence (LRFL) in unstained control and sample stained with ethidium homodimer. A cursor was set at the upper limit of the LRFL signal for unstained control, which was used as the "zero" channel for the stained cells within the sample. PDC: Percent Dead Cells.

clam using the same hemocyte:test particle ratio (1:10): 3 incubated with labeled bacteria (2 test, and 1 control well with Cytochalasin B added), and 3 incubated with standard fluorescent beads (2 test, and 1 control well).

The second experiment investigated the effect of live *V. tapetis* on hemocyte viability. Hemolymph samples were diluted with cold Alsever's solution (1:10, v:v) immediately after collection. Exponentially growing *V. tapetis* were suspended and diluted in sterile Alsever's solution to obtain about 10^7 cfu ml^{-1}. One milliliter of this suspension was transferred to a sterile plastic tube and mixed with 1 ml of diluted hemolymph to give approximately a 1:50, cell:bacteria ratio. Ten $\mu g\ ml^{-1}$ of Cytochalasin B was immediately added to the mixtures to prevent phagocytosis. They were then incubated with mild agitation on a rocker plate for 2 h at room temperature. A duplicate tube containing only sterile Alsever's solution and Cytochalasin B was prepared for each clam and was used as a control preparation. After incubation, EHD was added and the percentage of dead hemocytes measured using the flow cytometer as described previously.

Statistics

Percent phagocytosis and viability values were arcsine transformed before the use of statistical tests, however tables show means and standard errors of non-transformed values. Correlation analysis was used in the validation of the phagocytosis and viability assays. Correlation analysis, as well as repeated measures ANOVA, was also used to test the effect of storage time on the percent phagocytosis. Mean values in clams with (symptomatic) and without (asymptomatic) BRD symptoms were compared using a Student's *t*-test. This test was also used to compare the *in vitro* effect of *V. tapetis* on hemocyte viability. Differences were considered significant at $\alpha = 0.05$.

RESULTS

Assay Validation

Phagocytosis

The percent phagocytosis of beads calculated by flow cytometry (19.9 ± 2.5) was significantly ($P = 0.039$) lower than that calculated by fluorescent microscopy (26.4 ± 2.6), but the two methods were highly correlated (N = 24, $r^2 = 0.81$, $P < 0.0001$). Microscopic observation confirmed that the beads associated with hemocytes were internalized. The percent phagocytosis in duplicate wells was also highly and significantly correlated (N = 72, $r^2 = 0.67$, $P < 0.0001$); consequently, two wells per individual was considered sufficient replication in all subsequent experiments. Although there appeared to be a slight decrease in the percent phagocytosis in samples stored for seven days at 4°C (Table 1), a repeated measures ANOVA showed no statistically significant effect of time. Nevertheless, all further flow cytometric samples were processed within 24 hours because there was some microscopic evidence of an increase in broken cells and membrane fragments in stored samples. Fluorescence intensity (channel number) was equal to 5.7 ± 0.3 in non-phagocytic cells (mean ± SEM), 12.6 ± 4.1 in free beads, and 27.4 ± 7.3 in phagocytic cells. No significant changes in fluorescence intensity were observed in preserved samples.

TABLE 1.

Effects of storage on flow cytometric counts of percent phagocytosis in *R. philippinarum*. Samples were processed at the day of collection and re-analyzed after 3 and 7 days of storage at 4°C. Means, standard errors of the means, and correlation coefficients are presented (*N* = 24 clams with 2 replicates each). No significant differences were observed among means (repeated measures ANOVA) which were highly correlated (*P* < 0.0001).

	Day 0	Day 3	Day 7
Mean ± SEM	22.3 ± 1.3	18.8 ± 0.9	19.9 ± 1.1
Day 0	1	0.81	0.85
Day 3		1	0.77
Day 7			1

Viability

The percentage of dead hemocytes calculated by flow cytometry (3.7 ± 1.1) was not significantly different from values measured microscopically (4.2 ± 1.0, $P = 0.69$), and the two methods were highly correlated (N = 10, $r^2 = 0.92$, $P < 0.001$). About 99.9% of heat-killed hemocytes were located within the upper channels on the LRFL histograms, and thus detected by the flow cytometer as dead cells. Flow cytometric estimates indicated that the hemocyte mixture made without addition of heat-killed hemocytes contained 3.8% dead cells, while those containing 25, 50 and 75% heat-killed cells contained 28.1, 52.0 and 75.6% dead hemocytes, respectively.

Disease Effects on Phagocytosis and Hemocyte Viability

Flow cytometric measurements showed a somewhat lower percentage of phagocytic hemocytes (7.2 ± 1.0%, N = 21) compared with those from asymptomatic animals (10.4 ± 1.3%, N = 23, $P = 0.044$) and no difference in fluorescence intensity among phagocytic hemocytes (33.2 ± 9.0 for diseased and 25.1 ± 5.1 for asymptomatic clams, $P = 0.420$). Symptomatic clams had a significantly higher ($P = 0.0006$) percentage of dead hemocytes (13.4 ± 1.0%, N = 54) when compared with asymptomatic clams (9.2 ± 0.5%, N = 20).

Temperature Effects on Phagocytosis

The percent phagocytosis was clearly related to the temperature at which the hemocyte-bead mixture was held (Table 2). This

TABLE 2.

Effect of temperature on *in vitro* phagocytosis of beads by hemocytes from *R. philippinarum* (*N* = 24 clams with 2 replicates each). For each parameter, letters (a, b and c; or x and y) represent significant differences among different temperatures (ANOVA, *P* < 0.05). Fluorescence intensity refers to the peak fluorescent channel of those hemocytes that had phagocytosed beads.

	8°C	13°C	21°C
% Phagocytosis			
Mean ± SEM	7.9 ± 0.7[a]	14.9 ± 1.0[b]	21.1 ± 1.2[c]
Range	2.7–19.3	4.4–25.5	12.5–41.6
Fluorescence Intensity			
Mean ± SEM	15.1 ± 3.0[x]	24.0 ± 4.4[y]	29.6 ± 8.3[y]
Range	10–21	18–33	19–38

percentage at 21°C was about 21%, at 13°C, it was 15%, and at 8°C, it was 8%. Similarly, fluorescence intensity within phagocytic cells was also dependent upon the incubation temperature. This was about 30 and 24 in mixtures incubated at 21 and 13°C, respectively, and only 15 in those held at 8°C (Table 2).

Uptake of V. tapetis versus Beads

Clam hemocytes phagocytosed fluorescent latex beads at a significantly higher rate (22.3 ± 1.3%) than they did FITC-labeled V. tapetis (12.8 ± 2.9%, $P = 0.002$, N = 24).

Hemocyte Killing by V. tapetis

The percentage of dead cells increased significantly after incubation of hemocytes with the pathogenic bacterium, V. tapetis. In control mixtures, the percentage of dead hemocytes was 6.8 ± 2.2 while it reached 20.4 ± 5.9% in mixtures with V. tapetis added (Student's t-test, $P = 0.029$, $N = 11$).

DISCUSSION

Flow cytometry has been used in several previous studies to quantify phagocytosis and hemocyte viability in marine bivalves (Alvarez et al. 1989; Ashton-Alcox & Ford 1998; Brousseau et al. 2000; Fournier et al. 2001). However, none of these studies reported validating the methodology by comparing the flow cytometric results against standard microscopic measurements. In this study, we quantified phagocytosis and hemocyte viability in individual clams by both flow cytometry and microscopy. The high correlation between the two methods shows that flow cytometry is not only a rapid and versatile method for analyzing these two important parameters, but that the results are very comparable to more traditional methods for assaying marine bivalve hemocytes.

Notwithstanding the good correlation between flow cytometry and microscopy, differences in means between the two methods may be significant. Some of this disparity undoubtedly comes from uncertainty in drawing bitmaps to delineate presumed flow cytometer particle groupings. In our study, the fact that microscopy estimated a higher percent phagocytosis may be the result of the inclusion of some cell debris in samples analyzed by flow cytometry. Large, bead-free debris could fall above the noise discriminator for forward light scatter and be counted as non-phagocytosing cells, which would decrease the calculated percent phagocytosis. Indeed, microscopic observation revealed the presence of a limited quantity of such cell debris, which increased when samples were stored for several days. Such debris was not generated in the viability assay since the experimental protocol did not involve the addition of trypsin and the mild sonication used in the phagocytosis assay to detach adhered cells. Probably for this reason, the percentages of dead cells obtained using flow cytometry were not different from those measured microscopically.

Critical to phagocytic assays is the detachment of cells from the incubation vials and the separation of non-ingested particles from the phagocytic cells. Trypsinization is generally used to detach cells (Alvarez et al. 1989) while a variety of methods have been developed to discriminate non-ingested particles (De Boer et al. 1996; Mortensen & Glette 1996; Lopez-Cortes et al. 1999). We found that a single procedure, trypsinization in the presence of EDTA followed by gentle sonication, was quite effective in both detachment and separation steps. The centrifugation step over a sucrose gradient (Alvarez et al. 1989) is not required here to separate non-bound beads from phagocytic cells since the forward light

scatter allows easy differentiation between these particles based on size.

In this study, fluorescent latex beads were used as standard experimental particles for phagocytosis, as in the work of Alvarez et al. (1989) and Brousseau et al. (2000) who studied phagocytosis by hemocytes in Crassostrea virginica and Mya arenaria, respectively. These commercially available, fluorescent beads are extremely convenient for reproducible flow cytometric work due to their standardized sizes and fluorescence, qualities that are difficult to establish by labeling natural microorganisms. It must be recognized, of course, that latex beads may not evoke the same response from hemocytes as would foreign cells. Indeed, the present work demonstrated that the uptake of labeled V. tapetis was significantly lower than that of beads, despite precautions taken to ensure the same experimental conditions and hemocyte-to-particle ratios. Differences between the two test particles may be the result of specific interactions between hemocytes and beads or bacteria involving recognition factors that retard the uptake of formalin-fixed bacteria. Using microscopy, Lopez-Cortes et al. (1999) studied the phagocytic activity of R. philippinarum against V. tapetis and noted that the uptake of bacteria depended on the V. tapetis strain used. They also noted that viable V. tapetis were more efficiently phagocytosed than were the formalin-fixed bacteria and concluded that this was related to the presence of "specific recognition molecules" on the outer membrane of V. tapetis that combine with hemocyte receptors and that might be altered during fixation. Most work using labeled bacteria as test particles has been done after fixation of the microorganisms, which stabilizes the tag intensity because it prevents cell division or other processes that could alter intensity. Nevertheless, with appropriate controls, the use of live tagged bacteria should be included in cytometric assays to investigate the role of bacterial epitopes in recognition processes.

The development of BRD was associated with a significant decrease in phagocytic activity by hemocytes and an increase in the percentage of dead hemocytes. The lower phagocytic activity in diseased clams could be related to the percentage of dead hemocytes, since dead or moribund hemocytes are not capable of phagocytosis. Previous work has shown that clams with BRD have a high percentage of dead cells in the hemolymph compared to healthy animals and that this percentage increases with the development of the disease (Allam et al. 2000a, 2000b). The loss of phagocytic capacity and the death of hemocytes may result from deterioration of the physiological condition in severely infected clams (Plana et al. 1996), or from direct killing of hemocytes by V. tapetis. Indeed, results presented here demonstrate that V. tapetis is able to kill clam hemocytes in vitro.

It is often of interest to know the number of particles a phagocyte has ingested (phagocytic index) as well as the proportion of phagocytosing cells. The peak fluorescence intensity in the hemocytes that did ingest beads provided an index for the relative number of beads ingested by each cell. From this, it can be concluded that although clams with BRD symptoms had a smaller proportion of phagocytic hemocytes compared to asymptomatic clams, there was no difference in the number of beads each cell ingested.

Our results show a positive correlation between temperature and both percent phagocytosis and the phagocytic index of latex beads and agree with previous studies of other marine bivalves (Feng & Feng 1974; Foley & Cheng 1975; Alvarez et al. 1989; Tripp 1992; Chu & La Peyre 1993). It is relevant that the development of BRD seems to be at least partially controlled by water

temperature (Paillard et al. 1997; Allam 1998). Laboratory experiments have shown high prevalence and intensity of the disease when clams are incubated at 8°C and 13°C compared to clams incubated at 21°C (Allam 1998). Conversely, there are improved repair processes (recalcification) at 21°C as compared to the lower temperatures. It is possible that the low prevalence of BRD at 21°C is related to better performance of the clam's defense system, including phagocytosis. The pathogen may also be less virulent at this temperature, although growth of *V. tapetis* is not inhibited at 21°C (Maes 1992; Paillard et al., unpublished).

In summary, we have described and validated flow cytometric methods to measure the phagocytic activity and viability of hemocytes from the clam, *Ruditapes philippinarum*. Additional types of cytometric measurements will undoubtedly be adapted from vertebrate systems for use with molluscs and other invertebrates. For example, reactive oxygen species (ROS) generated by phagocytic cells represent a current concern of bivalve pathobi-

ologists and ecotoxicologists (Winston et al. 1996; Bramble & Anderson 1997; Lambert & Nicolas 1998). The successful use of flow cytometry for ROS measurements in aquatic mammals (De Guise et al. 1995) and fish (Verburg van Kemenade et al. 1994) indicate that flow cytometric methods can also be adapted for this purpose in bivalves. As new cytometric assays come into use, the developmental protocol should include some type of verification against a method that is considered the standard for that assay.

ACKNOWLEDGMENTS

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