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Phagocytic Ability of Oyster Hemocytes Measured by a Simple Method Using Bacterial Thin-layer

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Summary

A simple method called the phagocytic plaque assay was used to visually present and estimate the phagocytic ability of the hemocytes from the giant Pacific oyster, *Crassostrea gigas*, using adherent *Staphylococcus aureus* cells on a plastic dish. Many plaques appeared on the bacterial thin-layer when the oyster hemocytes were overlaid and incubated on killed cells of *S. aureus*. At both 20°C and 30°C, the phagocytic ability of the oyster hemocytes were more activated with a significantly larger average area of plaques than were the hemocytes at 10°C. This method was considered to be practical and effective evaluating the qualification of phagocytosis by the oyster hemocytes, however, some problems for the quantification analysis still remain to be solved.

Endocytosis is a fundamental cellular function in which vesicles and vacuoles formed by the plasma membrane regulate the uptake of molecules. Phagocytosis, one form of endocytosis, is used to describe the uptake of large particles. In mammals, many types of cells, especially the granular leucocytes and macrophages, are responsible for the phagocytosis of foreign particles (Silverstein et al., 1977). The size of particles phagocytosed by mammalian macrophages has been described as a range of 0.01 μm to 10 μm and the particles may include microorganisms such as viruses, bacteria and fungi (Cohn, 1972). Phagocytosis involves the disposal of these microorganisms ; therefore, phagocytic cells play an important role in the primary process of innate immunity in mammals.

The host-defense system of oysters as well as other bivalve molluscs is considered to mainly depend on circulating hemocytes that are present in the hemolymph. These cells possess a strong migration ability in response to invading microorganisms including potential pathogens, and subsequently, actively phagocytize these invaders (Fisher, 1986 ; Alvarez *et al.*, 1989 ; McCormick-Ray and Howard, 1991 ; Lopez-Cortes *et al.*, 1999 ; Takahashi and Mori, 2001). The

molluscan hemocytes share morphologic and functional similarities with mammalian phagocytic leukocytes (reviewed by Adema *et al.*, 1991; Cheng, 1981; 1996; 2000) and, like these leukocytes, have the ability to recognize, engulf, and internally degrade biological particles including foreign microorganisms (Foley and Cheng, 1975).

Several methods have been proposed to estimate the phagocytic abilities of the hemocytes in bivalve molluscs *in vitro*. In general, light microscopy has been the method of choice for the qualitative determination of particle uptake (reviewed by Robohm, 1984; Cheng, 2000). Bacteria, yeast cells and their derivative agents (e.g., zymosan) are often used as foreign particles, and are better visualized following staining under a light microscope. Although there are advantages to use the light microscope to observe ingestion in hemocytes, the interpretation of the qualitative and quantitative observations of phagocytosis is very difficult; e.g., the nonspecific attachment of bacteria to hemocyte surfaces is unable to differentiate from the active internalization by hemocytes. The flow cytometry technique is currently used in bivalve studies to assess the ability of phagocytic hemocytes (Ashton-Alcox *et al.*, 2000). Since flow cytometry can rapidly count a large number of cells and particles, application of this technique for quantitating particle uptake has a merit. In the study on the hemocytes from the clam, *Ruditapes philippinarum*, the flow cytometry results showed that the phagocytic percent toward fluorescent latex beads highly correlated with those obtained by fluorescent microscopy (Allam, unpublished; Ashton-Alcox *et al.*, 2000). Therefore, the flow cytometric analysis has been extremely useful in the measurement for the phagocytic ability of the bivalve hemocytes. However, the method requires special and expensive instruments such as a flow cytometer.

Seki et al. (1989) developed a method for measurement of the phagocytic ability of human leucocytes using a thin-layer of *Staphylococcus aureus* formed on a plastic dish. They designated this method as a phagocytic plaque assay. Phagocytic ability in the assay was evaluated as the width of the blank spaces (plaques) of a bacterial thin-layer by the ingestion of *S. aureus* cells by leucocytes. Seki *et al.* (1989) and Shinji *et al.* (1998) reported that the phagocytic plaque assay is simple and sufficiently reproducible for measurement of the bacteria uptake by human leucocytes under various conditions. In the study of leucocytes from carp *Cyprinus carpio* and rainbow trout *Oncorhynchus mykiss*, the method was also useful for quantification of the phagocytic ability (Nakayasu *et al.*, 1995). In the present study, therefore, to assess the phagocytic ability of the hemocytes from the giant Pacific oyster, *Crassostrea gigas*, I attempted to use the phagocytic plaque assay method.

Materials and Methods

Oysters

The specimens of the giant Pacific oyster, *C. gigas*, with an average shell height of 12.4 cm used in this study were obtained from a hanging-culture bed in Matsushima Bay, Miyagi Prefecture, Japan. They were then transferred to our laboratory and held in a 180-l aquaria with circulating, filtered artificial seawater (MARINE ART BR, Senju Seiyaku Co., Osaka, Japan) for 5-7 days. The water temperature was maintained at 15 ± 1°C.

Oyster hemocyte isolation

The hemocyte pools were obtained from three oysters. The hemolymph was withdrawn from the blood sinus in the adductor muscle using a tuberculin syringe with a 26 gauge, 0.5 inch needle. Each oyster was bled only once. The hemolymph was transferred to a 15-ml centrifuge tube and centrifuged at $290 \times g$ for 20 min at 4°C to separate the hemocytes. The resulting hemocyte pellet was washed three times with an ice-cold balanced salt solution for the oyster hemocytes (oyster BSS ; Takahashi and Mori, 2001) and the final hemocyte pellet was resuspended in the oyster BSS at 5×10^6 cells/ml. Viability of the hemocytes higher than 90% was observed on the basis of the dye exclusion test with 0.05% trypan blue in the oyster BSS. To minimize cell clumping, the hemocyte suspension was maintained on ice until used.

Bacteria and their cultivation

The bacterial strain tested, *S. aureus* IFO 3761, was obtained from the Institute for Fermentation, Osaka (Osaka, Japan). *S. aureus* was grown for 24 hrs in heart infusion broth (Difco Laboratories, Detroit, MI, USA) at 30°C. The grown cells were harvested by centrifugation at $4{,}450 \times g$ for 20 min and washed three times by centrifugation with 0.01 M phosphate-buffered 0.15 M saline (PBS, pH 7.4). The packed bacterial cells were suspended in PBS and killed by heating for 30 min at 100°C. The heat-inactivated cells were washed once with PBS and resuspended in oyster BSS. These *S. aureus* cells were used to form a bacterial-thin layer.

Formation of the bacterial-thin layer and estimation of phagocytic ability

Two milliliters of the *S. aureus* suspension (approximately 5×10^8 heat-inactivated cells/ml) was placed on a plastic dish for tissue culture (60 mm in diameter, Sumitomo Bakelite Co. Ltd., Tokyo, Japan). The dish was allowed to stand for 60 min at room temperature. After incubation, the supernatant in the dish was carefully removed with a Pasteur pipette without defacing the bacterial-thin layer formed on the bottom of the dish.

The phagocytic ability of the oyster hemocytes was tested at three different incubation temperatures. The hemocyte suspension (1 ml, 5×10^6 cells/ml in oyster BSS) was overlaid onto the bacterial-thin layer in the dish, and then incubated for 2 hrs at 10°C, 20°C, and 30°C. The phagocytic ability was also examined in three different incubation periods: 30 min, 60 min, and 120 min at 30°C. At the end of each incubation period, the supernatant was removed and washed by gentle shaking with oyster BSS. After washing, the formed hemocytes-bacterial-thin layer was air-dried and fixed with absolute methanol, and subsequently stained with Giemsa solution. Positive phagocytosis was represented as blank spaces (plaques) of the bacterial-thin layer caused by the phagocytic hemocytes. The micrographic images of the plaques with $\times40$ objective lens were recorded with a CCD camera (PDMC II/OL, Olympus Co., Tokyo, Japan), and subsequently the number and areas of the plaques were analyzed as the phagocytic ability using NIHImage software on a PC (developed at the US National Institute of Health). The phagocytic index (PI) was expressed as the average area (μm^2) of the plaques measured over 100 plaques in each experiment.

Statistical analysis

All data were presented as mean$\pm$standard error (SE) of the mean. A statistical analysis was performed using GraphPad Prism 4 software on a PC. The differences in the average area between the three temperatures groups and three different incubation period groups were compared using Tukey's multiple comparisons test. A p value of <0.05 was used to indicate significant differences.

Results and Discussion

To examine the phagocytosis of *S. aureus* cells by the oyster hemocytes, *in vitro* phagocytic plaque assay was conducted. Representative micrographs of the bacterial-thin layer and the plaques are shown in Fig. 1. The bacterial layer was well formed on the dish surface without empty spaces (Fig. 1A), whereas many plaques appeared on the bacterial thin-layer when the oyster hemocytes were overlaid and incubated for 120 min at 30°C (Fig. 1B). Microscopic observations showed that the plaques extended around the adhering oyster hemocytes. Therefore, the formation of the plaques was due to phagocytosis of *S. aureus* cells by oyster hemocytes.

The effects of three temperatures on the phagocytosis were tested (Fig. 2). The increase in temperature increased the width of the plaque area associated with the ingestion of bacteria by the hemocytes. At both 20°C and 30°C, the phagocytic ability of the oyster hemocytes was more activated with a significantly larger average area of plaques than that at 10°C. In a study of phagocytosis using flow cytometry, the phagocytic ability of hemocytes was positively correlated

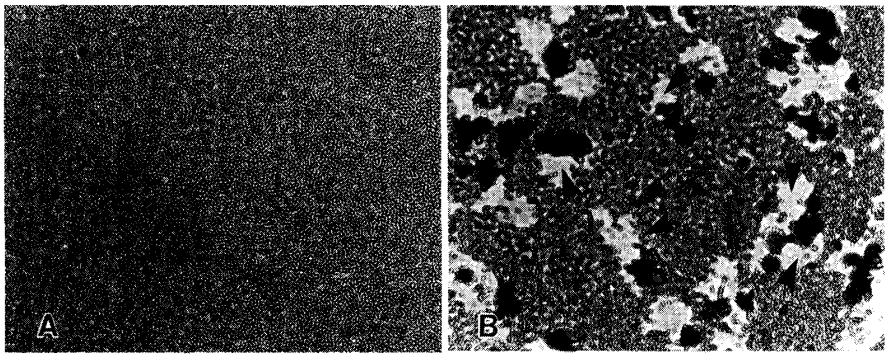

FIG. 1. Plaques formed on the bacterial-thin layer due to phagocytic ability of oyster hemocytes. *Staphylococcus aureus* was used to make the bacterial-thin layer (A, control). Many plaques (arrow heads) appeared on the bacterial-thin layer when hemocyte suspension was overlaid and incubated for 120 min at 30°C (B).

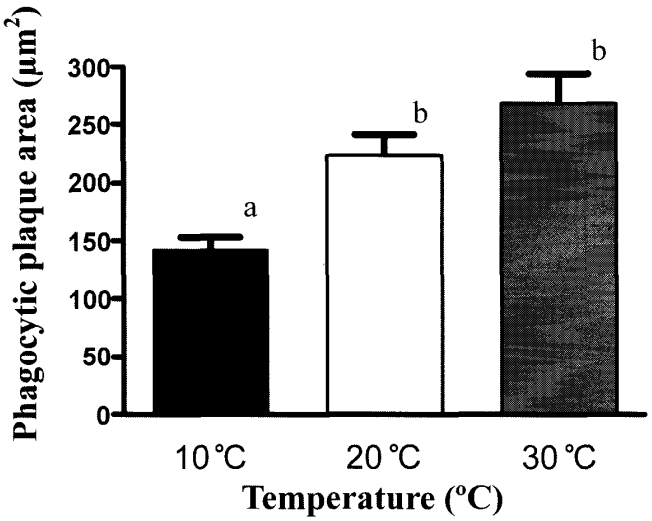

FIG. 2. Average area (μm^2) of phagocytic plaques made by oyster hemocytes at three different temperatures. Data are presented as mean$\pm$SE (n=8). A p value of <0.05 by Tukey's multiple comparisons test between the different temperature groups. Different letters denote significant differences.

with temperature over the range of 8°C to 37°C in the eastern oyster, *C. virginica* (Alvarez *et al.*, 1989). In microscopic studies, the phagocytic ability also revealed the maximal value at 37°C, while the hemocytes had no or low phagocytic ability at 4°C in the clam, Mercenaria mercenaria (Foley and Cheng, 1975) and in *C. virginica* (Rodrick and Ulrich, 1984). Based on a remarkable decrease in the phagocytic ability of molluscan hemocytes at lower temperatures, Foley and Cheng (1975) proposed that the adherence of hemocytes to bacteria did not occur to any significant extent at those temperatures. In the present study, it is considered that the enhancement of the phagocytic ability expressed as enlargement of the plaque areas resulted from activation of migration activity of

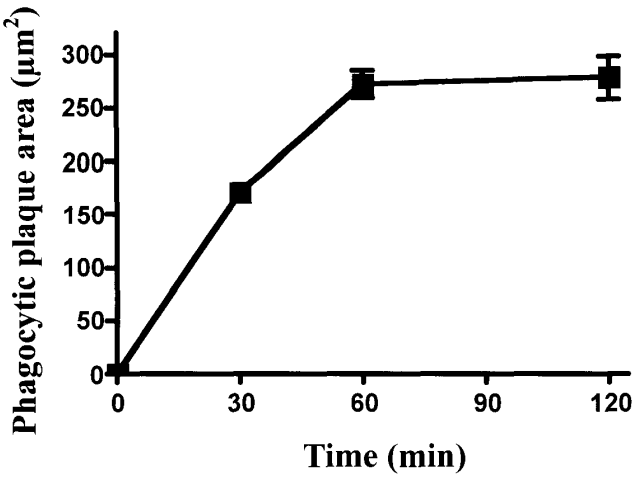

FIG. 3. Changes in average area (μm^2) of phagocytic plaques made by oyster hemocytes. Data are presented as mean±SE (n=5). A p value of <0.05 by Tukey's multiple comparisons test between the different incubation period groups.

the hemocytes.

Phagocytosis continued to increase for the first 1 hr of incubation period (Fig. 3), but average width of the phagocytic plaques did not extended after 1 hr.

The phagocytic ability is evaluated using two parameters: one is the phagocytic rate and the other is the phagocytic index. The phagocytic rate is calculated as the percent of hemocytes engulfing target particles among the total number of hemocytes tested. On the other hand, the phagocytic index is calculated as the number of internalized particles in each phagocytosing hemocyte. Accordingly, it is considered that the phagocytic rate represents the rate of hemocytes with phagocytic capability among the total hemocyte population, and the phagocytic index indicates the average individual phagocytic ability of the hemocytes. The phagocytic plaque assay used in this study was substituted by the total area of the plaques for the phagocytic rate, and the width of the plaque substituted with one hemocyte for the phagocytic index. However, it is considered that the phagocytic plaque assay is less accurate than the measurement of phagocytosis by light microscopy. In this study, one plaque was usually assigned to one hemocyte under lower temperature condition and shorter incubation time. On the other hand, fused larger plaques were observed at the higher temperature (30°C) or after longer incubation periods caused by two or more hemocytes, since the migration activity of the hemocytes was enhanced by the higher temperatures. To achieve accurate estimation of phagocytic ability by the plaque assay, either the test must be carried out at suitable temperature (20°C) for appropriate incubation time (30 min or 60 min).

In conclusion, the phagocytic plaque assay is convenient and available for the qualitative observation of bacteria uptake by oyster hemocytes, as the method

needs no special equipment or facilities, although some minor modifications are required quantification of the phagocytic ability.

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