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DEVELOPMENT AND EVALUATION OF TECHNIQUES TO STUDY ACQUIRED IMMUNITY TO *PERKINSUS MARINUS* IN THE OYSTER, *CRASSOSTREA VIRGINICA* (GMELIN)

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ABSTRACT This paper describes a radiometric technique developed to measure phagocytosis of *Perkinsus marinus* zoospores by oyster hemocytes. The spores of *P. marinus* were radiolabeled by culturing *P. marinus* presporangia and sporangia in estuarine water (22‰) containing ^{14}C-glycine. The percent of spores phagocytized by hemocytes was determined by the uptake of radioactivity by hemocytes.

Results from preliminary experiments to test the efficiency of using an osmotic infiltration method for immunizing oysters are also reported. It was found that oysters can take up both dissolved antigen (radiolabeled bovine serum albumin) and particulate antigen (^{14}C-labeled zoospore homogenate) through osmotic infiltration. The uptake of the antigen was correlated with the concentration of antigen added to the water but was not affected by water temperature.

KEY WORDS: Oyster, acquired immunity, techniques.

INTRODUCTION

Perkinsus marinus (Dermo) and *Haplosporidium nelsoni* (MSX) are two parasitic pathogens which have been destructive to estuarine oyster populations in the Middle Atlantic Region since the introduction of *P. marinus* in the 1950's and MSX in the 1960's. However, there are some oysters that have survived the invasion of these pathogens (Andrews 1968; Haskin and Ford 1979; Ford and Haskin 1986). Those oysters that survive the epizootics are believed to possess certain genetic, or physiological characteristics which make them less susceptible to the pathogens (Maryland Sea Grant 1983, National Fisherman 1983). Two hypotheses have been suggested for the occurrence of this resistance: (1) disease resistant oysters are physiologically or genetically different from non-resistant ones, and (2) disease-resistant oysters acquired immunity through early exposure to the pathogens. These two hypotheses are probably mutually inclusive.

Although evidence for the development of acquired immunity in molluscs is far from satisfactory, there are several interesting findings. In 1964, Michaelson (1964) reported the production of a microacidial immobilizing substance by snails infected with *Schistosoma mansoni*. Acton and Evans (1968) found that the bacterophage T2 was cleared more rapidly from oyster (*C. virginica*) hemolymph after secondary injection than after primary injection. Feng and Stauber (1968) suggested that the precipitous reduction in the number of *Hexamita* sp. in resistant oysters 8 days post-injection might be attributed to the presence of acquired immunity. Furthermore, Hardy et al. (1977) demonstrated that exposure of oysters (*C. gigas*) to bacteria stimulated an increase substantially in the titre of bacterial agglutinin.

Disease problems in oysters and other bivalve species have stimulated interest to determine the feasibility of inducing acquired immunity to the pathogen *P. marinus* in American oysters, *Crassostrea virginica*. Like other invertebrates, bivalve molluscs do not appear to possess immunoglobulins. Phagocytosis is the principle mechanism by which bivalve molluscs normally defend themselves against invading pathogens and foreign materials (Cheng and Rifkin 1970; Cheng 1981; Cheng 1983). The importance of phagocytosis in determining the outcome of a disease has been established (Metchnikoff 1893; Sindermann 1971). The phagocytic activity of the host to invading pathogen is correlated with the degree of resistance (McKay and Jenkin 1970). Resistance is decreased by a lowering of phagocytic activity (Aarum 1967). In order to test the efficacy of immunization with a possible *P. marinus* vaccine, a technique was developed to measure the phagocytosis of *P. marinus* zoospores by oyster hemocytes. This paper describes the radiometric technique developed for this purpose.

Osmotic infiltration is a practical mass-immunization method which was originally developed by Amend and Fender (1976) for immunizing fishes. This method is less stressful on the animal and less time consuming than individual inoculation. Antigens are infiltrated into fishes during immersion of the animal in a hyperosmotic solution containing the antigen (Antipa and Amend 1977; Croy and Amend 1977; Bowers and Alexander 1981). Lewis and his associates (The University of the Sea, Vol. 15, No. 1, 1982, Texas A&M University; The University of the Sea, Vol. 14, No. 3, 1981, Texas A&M University) also successfully immunized shrimp against bacterial diseases by placing shrimp in hyposmotic water containing antigen. Since the osmotic infiltration technique has proven to be very effective and successful for mass-vaccination of small fishes and shrimp, we wanted to evaluate the osmotic infiltration technique for immunization of oysters. To examine whether oysters can take up antigens by osmotic infiltra-

tion, experiments were performed to determine the uptake of radiolabeled bovine serum albumin (^{14}C-BSA, Molecular weight = 69,000 daltons) and radiolabeled zoospore homogenate. Preliminary results from these experiments are reported in this paper.

MEASUREMENT OF PHAGOCYTOSIS OF *P. MARINUS* ZOOSPORES BY OYSTER HEMOCYTES

A summary of the sequence and procedure developed to measure phagocytosis of *P. marinus* zoospores by oyster hemocytes is shown in Figure 1.

Immunization and Maintenance of Oysters

Oysters (*Crassostrea virginica* (Gmelin)) were collected from upstream bars of the James River in Virginia. In this area, oysters have been protected by Dermo and MSX diseases by low salinity (5–14‰) (Andrews and Hewatt 1957; Chu, unpublished data). MSX and Dermo are believed to be inactive in salinity below 15‰. There were 2 groups of oysters (15 oysters per group): immunized and sham control. Oysters of the immunized group were injected intramuscularly with formalin-killed *P. marinus* zoospores twice (2.0×10^8 zoospores/oyster in 0.1 ml estuarine water) at a one week interval. Each of the sham control oysters was injected with 0.1 ml estuarine water. Both immunized and sham control oysters were held in a trough (210 × 60 × 15 cm L × W × H) filled with filtered (10 μm, 1 μm Cuno cotton filters) pasteurized estuarine water. Water in the trough was changed every two or three days. An algal diet (*Tetraselmis suecica*) was added to the trough daily (500 ml of $1-2 \times 10^6$ cells/ml per day). Twenty-four and 48 hrs after the second immunization, blood samples were taken from oysters (7 oysters/group/time period) for phagocytic activity measurement.

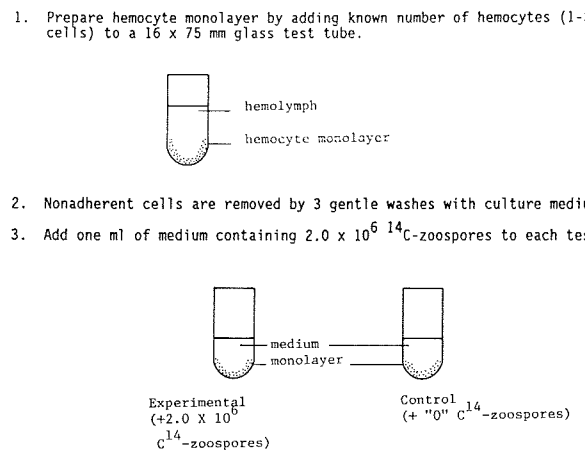

Figure 1. Procedure for quantifying phagocytosis of ^{14}C-zoospores of *Perkinsus marinus* by oyster hemocytes.

Collection of Oyster Hemocytes

Hemolymph was collected from oysters. A 27 gauge, 25 mm needle attached to a 1 ml sterile syringe was inserted in the adductor muscle of the oyster. Hemolymph was withdrawn and pooled at 4°C. The number of cells in the hemolymph was counted using a hemocytometer. About $1-2 \times 10^6$ cells in one ml of hemolymph were used for phagocytosis measurement.

Preparation of ^{14}C-labeled Zoospores

Presporangia and zoospores of *P. marinus* were cultured by methods described by Perkins and Menzel (1966). ^{14}C-labeled zoospores were obtained by culturing presporangia in 25 ml of 22‰ estuarine water containing 10 μCi ^{14}C-glycine (New England Nuclear, U.S.A.) at 27–28°C for 72–96 hrs. ^{14}C-labeled zoospores were harvested, treated with 0.3% formalin, and washed twice with sterile (0.22 μm filtered) estuarine water. ^{14}C-zoospores were then concentrated to a desired level for the phagocytosis study. The ^{14}C-zoospores obtained in this way contained $6.6-13.2 \times 10^{-2}$ dpm/spore. The percent of radioactivity leached from the zoospores after 24 hrs incubation in ^{14}C-glycine-free sea water was 0–25%.

Measurement of Phagocytosis

Known numbers of cells ($1-2 \times 10^6$ cells) in one ml of hemolymph were placed in 16 × 75 mm culture tubes and allowed to adhere at 15°C for 30 minutes. At the end of the time, nonadherent cells were removed by three gentle washes with minimal essential medium (MEM) and counted. About $1.5-2.0 \times 10^6$ ^{14}C-zoospores in 1 ml of MEM were added to the hemocytes and incubated at 15°C for 1.5 hours. Phagocytosis was stopped by discarding the supernatant and gently washing 3 times with MEM. Cell pellets were digested with 0.6 ml NCS (tissue solubilizer) at 50°C and the radioactivity in the aliquot was measured in 10 ml Aquasol with a scintillation counter. Percent of phagocytosis of ^{14}C-zoospores by oyster hemocytes was calculated using the following formula:

$$\%\ \text{of phagocytosis} = \frac{\text{CPM of hemocyte monolayer incubated with } ^{14}\text{C-zoospores} - \text{CPM of control hemocyte monolayer}}{\text{CPM } ^{14}\text{C-zoospores added to hemocyte monolayer.}}$$

Since the number of hemocytes placed in the glass tubes to prepare the hemocyte monolayer and the number of ^{14}C-zoospores added to the hemocyte monolayer are known, percent of phagocytosis can also be expressed in terms of number of ^{14}C-zoospores phagocytized by number of hemocytes. Counts of the number of nonadhering cells indi-

cated that about 95–97% of the hemocytes adhered. The phagocytic responses of hemocytes (2×10^6 cells) pooled from 7 immunized oysters and 7 sham control oysters employing this technique are shown in Figure 2. The results showed the uptake of ^{14}C-labeled zoospores by hemocytes sampled 24 and 48 hrs after the second immunization. The uptake of ^{14}C-labeled zoospores of *P. marinus* by hemocytes from immunized oysters was higher than hemocytes from control (non-immunized) oysters. It was speculated that a cellular response was elicited in oysters at 24 and 48 hrs after the second challenge with formalin-killed zoospores, but further study is needed to verify this speculation, and the specificity of the response has not been determined. The increased response of sham control at 48 hrs suggests a nonspecific reaction of oyster hemocytes.

Phagocytosis is usually measured by enumerating the number of hemocytes which have ingested bacteria (or the pathogen) or by measuring the optical density of abiotic particles (e.g. latex ring) ingested by oyster hemocytes (Anderson and Good 1976; Cheng and Sullivan 1984). The radiometric technique described in this paper is the first reported method to radiolabel an oyster pathogen and directly measure the phagocytosis of the radiolabeled pathogen by the oyster hemocytes. Phagocytosis includes processes of recognition, adherence, ingestion, destruction and disposal. Interaction of hemocytes and live or formalin-killed *P. marinus* zoospores has been examined with light phase contrast microscope, and both adhesion and ingestion were observed. It was assumed that the uptake of radioactivity by the oyster hemocytes was due to either adherence or ingestion. The application of this technique to measure phagocytosis of *P. marinus* spores by oyster hemocytes will be further evaluated.

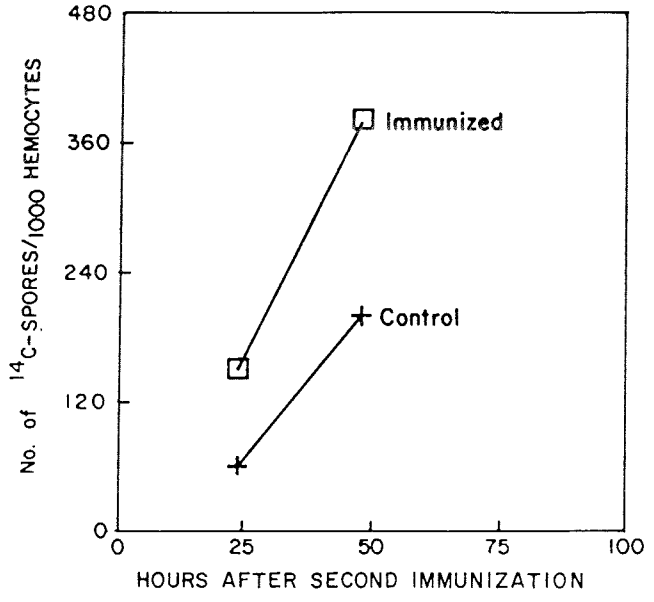

Figure 2. ***In vitro*** **cellular phagocytic response of oyster hemocytes.**

UPTAKE OF DISSOLVED AND PARTICULATE ANTIGENS THROUGH OSMOTIC INFILTRATION BY THE OYSTER, *C. VIRGINICA*

The size of oysters used for the osmotic infiltration experiments ranged from 2 to 3 cm shell height. Oysters were submerged individually for 1–3 hrs in hyposmotic water containing [^{14}C]-methylated bovine serum albumin (soluble antigen ^{14}C-BSA, molecular weight $\simeq$ 69,000 daltons, Amersham) or ^{14}C-labeled zoospore hemogenate (particulate antigen). The ^{14}C-labeled zoospore homogenate was prepared by disrupting the zoospores with sonifier cell disruptor (Model W185, Heat Systems—Ultrasonic Inc.). Hyposmotic water was prepared by lowering the salinity in which the oysters were held from 16‰ to 10‰ with chlorine free tap water. Uptake of ^{14}C-BSA by oysters submerged in water of the same salinity (16‰) was also determined. All the experiments were performed at room temperature ($\simeq$22°C), unless stated otherwise. To examine the effect of temperature on the uptake of antigens, the osmotic infiltration was performed at 18 and 30°C.

Results from the preliminary osmotic infiltration studies indicate that through osmotic infiltration the oyster can take up both ^{14}C-BSA and ^{14}C-labeled zoospore homogenate (Fig. 3 and Table 1). The uptake of antigen was correlated with the concentration of antigen added to the water (Figure 3). Elevated water temperature does not appear to increase but to decrease the antigen uptake; it was found that the uptake of C^{14}-BSA in oysters in water of 18°C was 2 times higher than in oysters in water of 30°C. A temperature of 30°C may stress the animals and retard the osmotic activity,

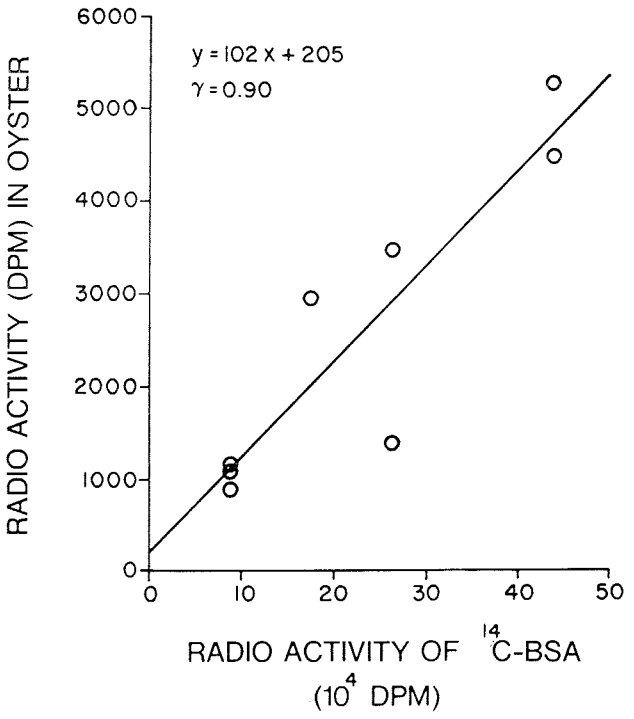

Figure 3. Uptake of ^{14}C-BSA by oysters through osmotic infiltration.

TABLE 1.

Uptake of radiolabeled zoospore homogenate by oysters*

Oyster	Radioactivity (DPM) of Zoospores Added to the Water	Radioactivity in Oysters (DPM)	% of Uptake
1	3896 (2.13 × 10^6 zoospores)	2022	51.9
2	3896 (2.13 × 10^6 zoospores)	1233	31.6
3	3896 (2.13 × 10^6 zoospores)	1875	48.3
$\bar{x}$ ± SD		1710.0 ± 419.5	43.9 ± 10.8
4	1047 (0.98 × 10^6 zoospores)	350	33.0
5	1047 (0.98 × 10^6 zoospores)	413	39.0
6	1047 (0.98 × 10^6 zoospores)	288	27.5
7	1047 (0.98 × 10^6 zoospores)	244	23.0
$\bar{x}$ ± SD		328.8 ± 73.7	30.6 ± 6.9

* Oysters were exposed to 2 different concentrations of ^{14}C-labeled zoospores from 2 different culture stocks at room temperature (22°C); exposure time was two hours.

TABLE 2.

Uptake of ^{14}C-BSA by oysters incubated in hyposmotic (10‰) and isosmotic (16‰) waters*

Oyster	Radioactivity (DPM) of ^{14}C-BSA	Radioactivity in Oysters	
		10‰	16‰
1	2.6 × 10^5	5738	
2	2.6 × 10^5	2830	
3	2.6 × 10^5	3922	
4	2.6 × 10^5		3297
5	2.6 × 10^5		4380
6	2.6 × 10^5		3373
7	2.6 × 10^5		1598
$\bar{x}$ ± SD		4163 ± 1469	3162 ± 1154

* Oysters were incubated at room temperature (22°C) for 3 hrs.

since the oysters were held in ambient water of 18–20°C prior to the experiment. Results also demonstrated that ^{14}C-BSA infiltrated into oysters held in water of the same osmotic concentration (Table 2). The uptake of C^{14}-BSA was similar to those immersed in hyposmotic water. The efficiency and reliability of the osmotic infiltration technique for immunization of oysters will need further investigation. The uptake of antigen in oysters could be the results of the synergy of osmotic infiltration, active transport (pinocytosis) and phagocytosis processes.

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