Occurrence and Characterization of the Defense-Factors in the Japanese Oyster Crassostrea gigas

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Occurrence and Characterization of the Defense-Factors in the Japanese Oyster *Crassostrea gigas*

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Summary

The occurrence and partial characterization of hemagglutinin, bactericidin and phagocytic capacity in the Japanese oyster, *Crassostrea gigas*, were investigated. High natural hemagglutinin activity was observed in the hemolymph, body fluids and tissue fluids. A variability in the titer was found when red blood cells of different origin were used, but specificity for the human blood group was not observed. The hemagglutinin was heat-labile, required calcium ions and declined when the pH of the medium was changed to the acidic side. The results of adsorption tests and inhibition tests showed that the oyster might have some types of hemagglutinins which possess different binding specificities. The natural bactericidal activity against *Arthrobacter* sp. was detectable in the oyster tissue extracts and was affected by the pH of the medium. There was no seasonal relationship between the natural bactericidal activity and the physiological activity. The oyster amoebocytes phagocytosed formalinized red blood cells *in vitro*. The mean percentage of amoebocytes phagocytosing 3 or more red blood cells was increased when these red blood cells were pretreated with the oyster hemolymph or body fluid. Finally, the possibility of expressing the physiological activity in terms of values of these factors is discussed.

Specific adaptive immunity in vertebrates is characterized by the production of specific antibodies. Invertebrates, however, possess neither antibodies nor complement system. Therefore, the internal defense system in invertebrates has been considered to be composed of non- or broad-specific factors.

In mollusks, as well as in other invertebrates, hemagglutinins (1-4), bactericidins (1, 2, 5, 6) and phagocytic capacities (7, 8) have been investigated in relation to their defense mechanisms and commonly designated as non-specific

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defense factors. The hemagglutinins and bactericidins have been detected in the hemolymph and certain organs of several invertebrate species (9). In some of these species, it has been reported that hemagglutinins may act as opsonins and enhanced the capacity for recognition of foreign particles and also that bactericidins may take part in the defense reaction against infections of bacteria and other potential pathogens (9). The experiments directed to understanding the roles of lysosomal enzymes released from circulating hemocytes, especially granulocytes, of gastropods and bivalves as a result of a challenge with exogenous nonself materials have been carried out from the viewpoint of elucidating the evolution of cellular immunity, including one of the defense factors, i.e., phagocytic capacities (8).

In gastropods, especially in fresh-water snails, a comparatively large number of studies have been made on the internal defense mechanisms. In bivalves, on the other hand, there have been only a few reports in this field of research. This is because snails can be easily bled and maintained in the laboratory, but bivalves can not (10). Therefore, it seems to be of great importance to obtain detailed information on the occurrence and characterization of the above-mentioned defense factors from the standpoint of comparative immunology. In addition, such information is necessary for evaluation of the activities of these factors in the context of the total defensive capacity, i.e., the physiological activity of the animal. The occurrence of hemagglutinin and bactericidin activities in the tissue extracts of the Japanese oyster, *Crassostrea gigas*, has been revealed through our previous study (2). The present study deals with the occurrence of oyster hemagglutinin activities in such preparations other than tissue extracts as hemolymph, pericardial fluid and others, together with partial characterization of agglutinins and bactericidins. Furthermore, the phagocytic capacities of oyster amoebocytes from the hemolymph is also reported in the present paper.

Materials and Methods

The experimental animals used in the present study were 2-year-old oysters, *C. gigas*, in hanging cultures in Miyagi Prefecture, Japan.

Hemagglutinin activities in tissue extracts, hemolymph, body fluid, tissue fluids and mantle cavity seawater were determined by the Cooke Microtiter System (Cooke Engineering Co., U.S.A.). The methods for preparing both tissue extracts and suspension of red blood cells (RBC) for hemagglutination test have previously been described (1). Hemolymph was withdrawn by syringe from the heart, and pericardial fluid was collected by Pasteur pipette from the pericardial cavity. Tissue fluids were obtained by centrifuging minced tissues. Mantle cavity seawater was sterile seawater (hemagglutinin titer : 0) that was poured into oyster mantle cavity (which had been exposed beforehand by cutting off the lower end of the right valve by means of a small handsaw) after washing with the same

sterile seawater. Hemagglutinin titer was recorded as the reciprocal of the last dilution of the sample giving a positive hemagglutination.

To examine the effect of temperature on the stability of oyster hemagglutinin, hemolymph and body fluid were incubated at 20-80°C for 30 min. After incubation, samples were centrifuged at 27,000×g for 20 min at 4°C. Hemagglutinin activity of the supernatant was assayed. For adsorption test, RBC were washed in 0.05 *M* Tris-HCl buffer solution (pH 8.0) containing 0.15 *M* NaCl and 0.01 *M* $CaCl_2$ by centrifugation. Equal volumes of oyster body fluid and packed RBC were incubated at 20°C for 1 hr. For inhibition test, 50 μl of 0.05 *M* Tris-HCl plus 0.15 *M* NaCl containing an appropriate concentration of test agent was added to each well after two-fold dilution. Following 1 hr incubation at 20°C, RBC suspension was added for hemagglutination assay.

The methods for determining the bactericidal activities in bivalve molluscan tissue extracts have been described in our previous reports (1, 2).

The culture medium and procedure for measuring oyster phagocytic capacities are shown in Table 1 and Fig. 1.

Results and Discussion

Occurrence of Defense-Factors and Their Partial Characterization

1. Hemagglutinins in the oyster

The extracts of the gill and digestive diverticula exhibited a high level of natural hemagglutinin activity against sheep red blood cells (SRBC) (2). Lower, but still considerable, activities were found in the extracts of the mantle and adductor muscle. The hemolymph showed a much higher natural hemagglutinin activity than was observed in the extracts of the gill and digestive diverticula collected at the same time (Table 2). About the same level of activity as found in the pericardial fluid was detectable in the body fluid and tissue fluids (Table 3). In this table, body fluid-I refers to the fluid oozing out from the adductor

TABLE 1. *Composition of oyster hemolymph medium (OHM)*

Constituent	g/liter
NaCl	27.0
KCl	0.7
$CaCl_2$	1.2
$MgCl_2 \cdot 6H_2O$	4.6
$NaHCO_3$	0.5
Na_2HPO_4	0.01
Glucose	1.0
Phenol red (0.4%)	1 ml/liter

Adjust to pH 8.0 with 1N HCl.

TABLE 2. *Hemagglutinin activities in oyster hemolymph, gill and D.d.* (Dec. 1980)*

Origin	Hemagglutinin titer
Hemolymph	304.0±60.3 (16)
Gill extract	80.0±10.1 (16)
D.d. extract	32.4± 8.5 (9)

* Digestive diverticula

1.5% SRBC were used as indicator cells. Means and standard errors (number of oysters) are shown.

Collection of hemolymph from the ventricle of oyster with a tuberculin syringe
↓
25 μl of hemolymph to a coverslip
↓
Preincubation in humidified chamber for 45 min
↓
Addition of 25 μl of 5% sheep erythrocytes (formalinized) in OHM
↓
Incubation for phagocytosis
↓
Washing with 125 μl of OHM
↓
Fixation in MeOH for 5 min
↓
Drying in the air
↓
Staining with May-Grünwald's solution for 10 min
↓
Staining with Giemsa's solution for 60 min
↓
Washing with OHM and D.W.
↓
Drying in the air
↓
Microscopy

FIG. 1. Outline of the method for measuring the phagocytosis of formalinized sheep erythrocytes by oyster amoebocytes *in vitro*.

TABLE 3. *Agglutinin activity in body fluid and tissue fluids of oyster (Dec. 1981)*

	Agglutinin titer*1	
	Range	Mean±S.E.*2
Pericardial fluid	32−256	124.5±34.7
Body fluid-I	32−256	120.0±22.7
Body fluid-II	32−512	147.9±70.0
Gill fluid	32−512	152.1±68.7
Mantle fluid	32−512	188.4±63.5
D.d.*3 fluid	16−256	90.9±42.8

*1 2% SRBC were used as indicator cells.
*2 Means and standard errors on 9 samples are shown.
*3 Digestive diverticula.

muscle injured when the right-valve was removed and body fluid-II stands for the fluid exuding after the operative removal of the gills and mantles, implying that a part of the remaining fluid-I was unavoidably mixed with fluid-II. In addition to the interior of the soft body, the natural hemagglutinin titer was detected in the

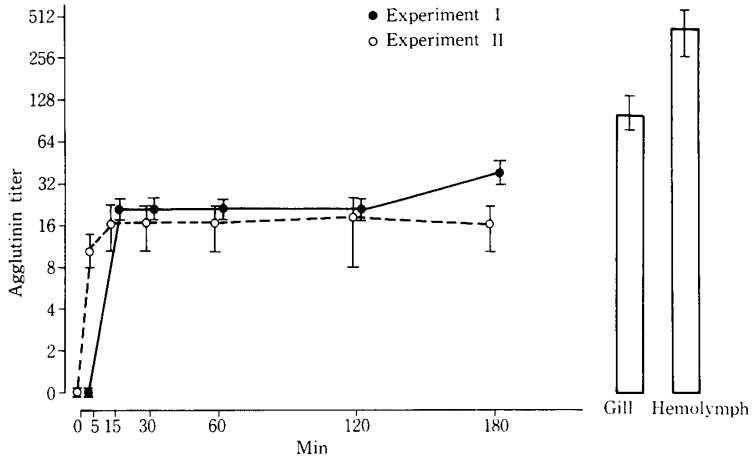

FIG. 2. Natural hemagglutinin activity of sterile seawater after being poured into the oyster mantle cavity (Feb., 1981). Sterile seawater (5 ml) was poured into the mantle cavity and 0.4 ml was collected at each time interval (0.4 ml of sterile seawater was added to the mantle cavity after each sampling). The hemagglutinin titers of the gill and hemolymph from the tested animals in Experiment I are shown on the right. 1% SRBC were used as indicator cells.

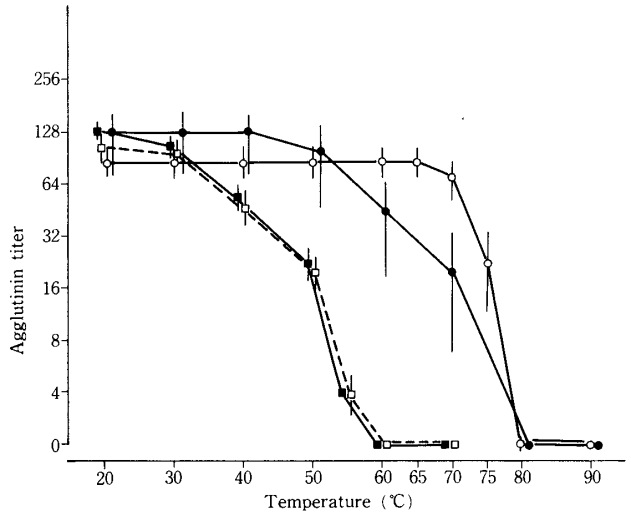

FIG. 3. Heat stability of hemagglutinin activity *in vitro* in oyster body fluid (Feb., 1982). 2% SRBC were used as indicator cells. Each point (filled circles) indicates the mean value for individual determinations on 5 samples (±S.E.). Heat stability of hemagglutinin activities *in vitro* of the gill (filled squares) and digestive diverticula (open squares) extracts and the hemolymph (open circles) is also presented for comparison.

seawater which was poured into the oyster mantle cavity, although it was lower than that of the gill or hemolymph from the animals tested (Fig. 2).

Heat stability of hemagglutinin activity *in vitro* in oyster body fluid-II (Table 3) was investigated and compared with the results of the tissue extracts and

hemolymph (Fig. 3). The stability in the body fluid was approximately intermediate between those of the tissue extracts and hemolymph. The low heat stability observed in the tissue extracts is supposed to be due to the denaturation at a relatively low temperature of proteins other than hemagglutinin(s). Measurement of natural hemagglutinin activities in the oyster body fluid-II against equine, sheep and human red blood cells revealed a markedly high activity against equine red blood cells (ERBC) (mean hemagglutinin titer: 973) and no significant difference in activity against three different types of human red blood cells (Table 4). It is suggested that the hemagglutinins of *C. gigas* body fluid lack human blood group specificity. Hemagglutinin activities against equine and human red blood cells disappeared after the addition of EDTA or sodium citrate (Table 5). They were restored by the addition of $CaCl_2$, but $MgCl_2$ had practically no recovering effect, indicating that calcium ions play an important role in the binding of hemagglutinins to these red blood cells. On the other hand, the activities against sheep red blood cells were maintained constant even when

TABLE 4. *Hemagglutinin activity in oyster body fluid against equine, sheep and human erythrocytes*

Type of erythrocyte	Hemagglutinin titer
	Mean±S.E.
Equine	972.8±307.2
Sheep	128.0± 35.0
Human (A)	83.2± 19.2
Human (B)	65.6± 27.7
Human (O)	76.7± 21.7

2% (v/v) erythrocytes were used as indicator cells.
N=7

TABLE 5. *Effect of divalent cations on hemagglutinin activity in oyster body fluid*

Two-fold dilution with TBS containing:	Hemagglutinin titer		
	Type of erythrocyte		
	Equine	Sheep	Human (O)
10 m*M* $CaCl_2$	1024	128	64
10 m*M* $MgCl_2$	256	128	64
20 m*M* EDTA	<2	128	<2
20 m*M* Na-citrate	<2	128	<2
20 m*M* EDTA, 30 m*M* $CaCl_2$	1024	128	64
20 m*M* EDTA, 30 m*M* $MgCl_2$	<2	128	<2

TBS: Tris-HCl (pH 8.0) containing 150 m*M* NaCl.
2% (v/v) erythrocytes were used as indicator cells.

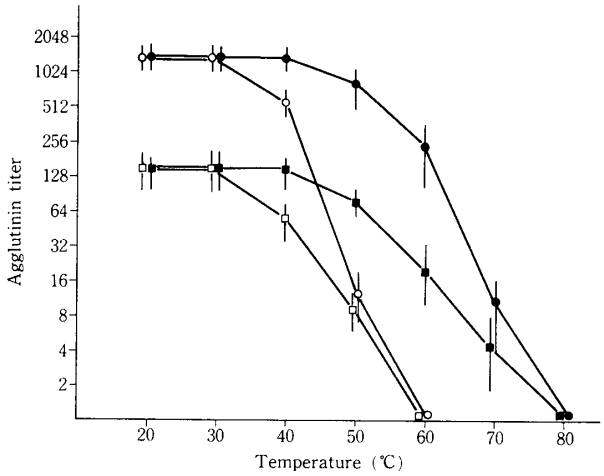

FIG. 4. Effects of divalent cations on the heat stability of hemagglutinin activities in the oyster body fluid (Feb., 1982). To samples were added equal volumes of TBS-10 m*M* $CaCl_2$ (pH 8.0) or TBS-20 m*M* EDTA (pH 8.0) ; then the samples were incubated at each temperature for 30 min. For the hemagglutinin assay, 2% SRBC and ERBC were used as indicator cells. Each point represents the mean value (±S.E.) on 5 samples.
Filled circles : plus TBS-10 m*M* $CaCl_2$ with ERBC
Open circles : plus TBS-20 m*M* EDTA with ERBC
Filled squares : plus TBS-10 m*M* $CaCl_2$ with SRBC
Open squares : plus TBS-20 m*M* EDTA with SRBC

EDTA or sodium citrate were added. These data were compatible with the results of McDade and Tripp (11). Heat stability of natural hemagglutinin activities in body fluid-II against ERBC and SRBC in the presence of $CaCl_2$ was greatly diminished by the addition of EDTA (Fig. 4), suggesting the possibility that calcium ions are involved in stabilizing the molecular structure of hemagglutinins in the oyster. In order to examine the pH stability of the oyster natural hemagglutinin activity, SRBC were used as indicator cells, since it was found that the hemagglutination of SRBC in the body fluid is not affected by the presence or absence of calcium ions (Table 5). The hemagglutinin activities were measured over a pH range of 6.0 to 9.5 by using three types of buffer solutions (Fig. 5). As a result, it was observed that the activities declined markedly when the pH was changed to the acidic side of physiological pH. The hemagglutinin titers at the pH below 6.0 could not be determined, owing to an intense hemolysis of indicator cells.

Cross adsorption tests (Table 6) indicated that oyster hemagglutinins for any type of human red blood cells employed were completely adsorbed with any type of human red blood cells, showing the lack of ABO blood-group specificity. However, such a complete adsorption was not found among three different species. This incomplete adsorption was most remarkable between human and equine red blood cells. None of the saccharides tried inhibited the hemagglutination of

ERBC by oyster body fluid, while N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and N-acetylneuraminic acid were specific inhibitors of oyster hemagglutinins for sheep and human red blood cells (Table 7). Both porcine gastric mucin (PGM) and bovine submaxillary gland mucin (BSM) were found to inhibit the hemagglutination of equine, sheep and human red blood cells by oyster body

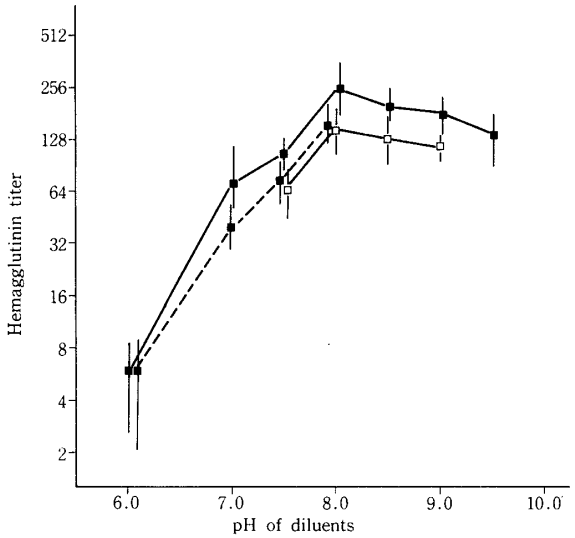

FIG. 5. Effects of pH on hemagglutinin activity of oyster body fluid (Aug., 1981). Samples were titrated using the following diluents;
Open squares: 50 m*M* Tris-HCl, 150 m*M* NaCl, pH 7.5-9.5
Filled squares (dotted line):
50 m*M* KH_2PO_4-50 m*M* Na_2HPO_4, 150 m*M* NaCl, pH 6.0-8.0
Filled squares (solid line):
Britton-Robinson buffer, 150 m*M* NaCl, pH 6.0-9.5
2% SRBC were used as indicator cells and suspended in the same buffer used as diluents. Each point represents the mean value (± S.E.) on 5 samples.

TABLE 6. *Hemagglutinin titers of oyster body fluid before and after adsorption with equine, human and sheep erythrocytes*

Adsorbed with erythrocyte of:	Hemagglutinin titer				
	Type of erythrocyte				
	Equine	Sheep	Human (A)	Human (B)	Human (O)
Equine	0	32	128	64	128
Sheep	8	0	16	16	16
Human (A)	1024	8	0	0	0
Human (B)	512	16	0	0	0
Human (O)	512	16	0	0	0
Unadsorbed	1024	128	128	128	128

2% (v/v) erythrocytes were used as indicator cells.

TABLE 7. *Effects of saccharides on hemagglutinin activity in vitro of oyster body fluid*

Saccharide added	conc. (m*M*)	Hemagglutinin titer		
		Type of erythrocyte		
		Equine	Sheep	Human (O)
D(+)-Glucose	500	1024	256	128
D(+)-Galactose	500	1024	256	128
D(+)-Mannose	500	1024	256	128
D(+)-Xylose	500	1024	256	128
L(−)-Fucose	500	1024	256	128
D(+)-Glucosamine	500	1024	256	128
D(+)-Galactosamine	500	1024	256	128
N-Acetyl-D-glucosamine	500	1024	32	4
N-Acetyl-D-galactosamine	100	1024	64	16
N-Acetylneuraminic acid	100	1024	64	4
None	—	1024	256	128

2% (v/v) erythrocytes were used as indicator cells.

TABLE 8. *Effects of PGM*[*1] *and BSM*[*2] *on hemagglutinin titer of oyster body fluid*

Mucin-Conc. (μg/ml)	Hemagglutinin titer		
	Type of erythrocyte		
	Equine	Sheep	Human (O)
PGM— 10	1024	256	128
— 50	1024	256	128
—100	1024	256	32
—200	512	128	16
BSM— 1	1024	256	8
— 5	1024	256	2
— 10	1024	128	<2
— 50	512	128	<2
—100	256	64	<2
None —	1024	256	128

*1. Porcine gastric mucin.
*2. Bovine submaxillary gland mucin.
2% (v/v) erythrocytes were used as indicator cells.

fluid (Table 8). BSM was more effective in inhibiting the hemagglutination than PGM. The most striking inhibition by BSM was observed in the hemagglutination of human red blood cells. From the results of adsorption experiments (Table 6) and inhibition tests (Tables 7 and 8), it may be concluded that *C. gigas* has at least 3 types of natural hemagglutinins which possess their individual binding

specificity for saccharide moieties. It can be assumed that severe inhibition of the hemagglutinin specific for human red blood cells by treatment with BSM is due to the fact that BSM is rich in N-acetylneuraminic acid. Hardy *et al.* (12) have isolated 7 types of hemagglutinins from *C. gigas* hemolymph by affinity chromatography using a Sepharose-BSM column.

TABLE 9. *Natural bactericidal activity in the extract of oyster digestive diverticula against test bacteria (Jan. to Feb. 1981)*

Test bacteria	Bactericidin titer*
Arthrobacter sp. HS 29 strain	352±175 (5)
A. citreus IFO 12957	37± 13 (6)
A. globiformis IFO 12136	0 (5)
Micrococcus luteus IFO 3333	284± 86 (9)
Escherichia coli NIHJ	0 (5)

* Reciprocal of dilution killing 50% of bacteria in 90 min at 20°C (per gram of original tissue).
Averages and standard deviations are presented.
Sample number is in parentheses.

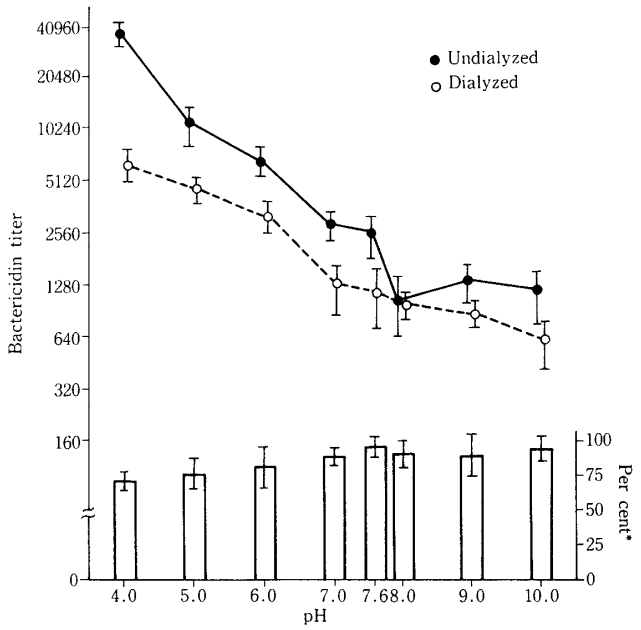

FIG. 6. Effects of pH on the natural bactericidal activity *in vitro* of the extract of oyster digestive diverticula against *Arthrobacter* sp., HS 29 strain. Each point indicates the mean value for individual determinations on 5 samples (±S.E.).

*Survival rate of HS 29 strain after incubating at each pH value for 90 min.

2. Bactericidins in the oyster

Arthrobacter sp. (HS 29 strain) and *Micrococcus luteus* were found to be adequate test organisms for the bactericidal activities of the oyster (Table 9). The natural bactericidal activity against HS 29 strain was detectable in the mantle and gill extracts in addition to the extract of digestive diverticula (2). The activity increased with the decrease of the pH of the extract of digestive diverticula from 7.0 to 4.0, while it was almost unchanged in the pH range from 7.0 to 10.0 (Fig. 6). In general, the dialysis caused a decrease in the level of bactericidal activity in the pH range tested.

3. Phagocytic capacities in the oyster

Formalinized SRBC were phagocytosed by 11% of the oyster amoebocytes *in vitro* after 60 min incubation at 20°C (Table 10). The maximal phagocytosis was obtained when the SRBC were treated with OHM (Table 1) buffered to pH 7.0-8.0 (Table 11). The mean percentage of amoebocytes phagocytosing 3 or more red blood cells significantly increased when these red blood cells were pretreated with

TABLE 10. *Phagocytosis of formalinized sheep erythrocytes by oyster amoebocytes in vitro: Time course change*

	Incubation time (min) at 20°C			
	15	30	60	90
% amoebocytes showing phagocytosis±SE	4.9±0.6	7.5±1.2	11.4±1.1	10.0±0.9
% of phagocytic amoebocytes containing:				
1 erythrocyte	87.2	85.8	83.0	91.5
2 erythrocytes	8.7	9.7	13.8	7.7
≧3 erythrocytes	4.1	4.1	3.2	0.9

TABLE 11. *Phagocytosis of formalinized sheep erythrocytes by oyster amoebocytes in vitro: Effect of pH*

	pH				
	5.0	6.0	7.0	8.0	9.0
% amoebocytes showing phagocytosis±SE[a]	3.0±0.3	3.8±0.1	5.3±0.6	5.5±0.6	3.2±0.1
% of phagocytic amoebocytes containing:					
1 erythrocyte	100	100	96.2	88.5	96.4
2 erythrocytes	0	0	3.8	10.7	0
≧3 erythrocytes	0	0	0	0.8	3.6

[a] Incubation for phagocytosis: 30 min at 20°C.

TABLE 12. *Phagocytosis of formalinized sheep erythrocytes by oyster amoebocytes in vitro : Effect of treatment with humoral factors*

Treatment of sheep erythrocytes with :	% amobocytes showing phagocytosis±SE	% of phagocytic amoebocytes containing :		
		1 erythrocyte	2 erythrocytes	≧3 erythrocytes
Hemolymph	11.5±0.6	52.4	25.1	22.5
Pericardial fluid	9.3±0.5	55.6	18.3	26.1
Body cavity fluid	7.3±0.6	28.8	17.4	53.7
Control (OHM)	9.3±0.3	76.0	18.0	6.0

oyster hemolymph or body fluids (Table 12), suggesting that the humoral factor(s) serving as opsonic substance(s) occurs in the oyster.

Possibility of Expressing the Physiological Activity in Terms of a Defense-Factor Value

Several long-term experiments were performed to ascertain whether there is a seasonal relationship between the so-called physiological activity (13-15) and the defense-factor activities in marine bivalve mollusks under culture.

It is well known that the physiological activity in the oyster, *C. gigas*, in hanging cultures with progressive development of the gonads is minimal at spawning (13). The natural hemagglutinin activities in the extracts of gill and digestive diverticula declined as sexual maturation proceeded, and fell to a minimum level just before and during spawning (2), paralleling the declines in the physiological activity of the animal. On the other hand, there was no seasonal relationship between the natural bactericidal activity of the extract of oyster digestive diverticula (Fig. 7) and the physiological activity. In addition, variations in bactericidal activity between individual members were found to be generally considerable, suggesting that the sensitivity of the test bacteria for bactericidal activity is not always stable enough to ensure a constant use of them (Fig. 7).

The bactericidal activity in the extract of digestive diverticula from 2-year-old oysters was observed to increase after the injection of formalin-killed HS 29 strain vaccine which was given into the connective tissue surrounding digestive diverticula (dose : 1.8×10^8 cells/g fresh soft-body wt.) or the adductor muscle (8.3×10^5 cells/g fresh soft-body wt.) (2). The hemagglutinin activities in the extracts of oyster digestive diverticula and gill were enhanced by the injection of 0.1 ml/oyster of 10% SRBC suspension into the connective tissue surrounding digestive diverticula (2).

The above results clearly exemplify the induction of higher defense-factor values by contact between the defense system and foreign materials in marine bivalve mollusks. It is possible that such immune responses may give rise to a danger of overestimating the actual defensive capacity of the animal when the

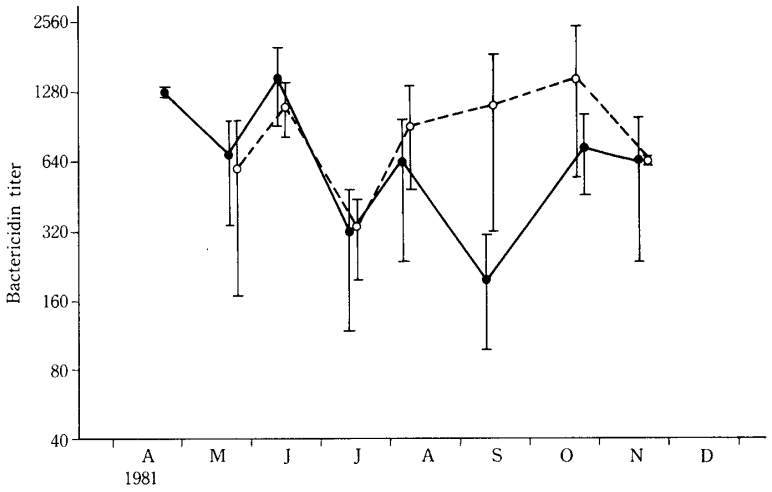

FIG. 7. Seasonal changes in the natural bactericidal activity of the extract of oyster digestive diverticula. Each point represents the mean value for individual determinations on 5 oysters (±S.D.). Open circles: tested against *Arthrobacter* sp., HS 29 strain; Filled circles: tested against *Micrococcus luteus*.

defense-factor value is used practically as a means of evaluating the physiological activity. Hence, it is necessary for us to suspect that heteroantigens have more or less invaded the animal when an unusually high defense-factor value is obtained during a series of immunological tests for the physiological activity.

It is reasonable to conclude that the effectual defense-factor as a practical means of evaluating the physiological activity is natural hemagglutinin(s) in the oyster. The phagocytic capacity in the oyster is also effectual in estimating their respective physiological activities in cases in which the values of these defense-factors can be easily determined by means of such a simple method as is usually employed by us. However, the bactericidal value is not available for practical use as an indicator for the physiological activity unless a constant use of bactericidal test bacteria showing high and stable sensitivity is ensured.

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