INDUCTION OF AN ANTIBACTERIAL ACTIVITY IN THE HEMOLYMPH OF THE MANILA CLAM, *RUDITAPES PHILIPPINARUM*

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ABSTRACT

In common with many invertebrates, host defense in bivalves is based largely on non-specific defense activities of circulating hemocytes and humoral factors present in the plasma. The understanding of these defense factors might allow us to manipulate the resistance or tolerance of bivalves to diseases. The present work focused on the effect of the challenge with a pathogen (the bacterium Vibrio tapetis) on antibacterial defense parameters in the hemolymph of its host (the clam Ruditapes philippinarum). Results indicate significant increase in total hemocyte counts and the induction of an antibacterial activity in the hemolymph of challenged animals. These results were discussed with emphasis on the presence in bivalve molluscs of antibacterial peptides close to those found in arthropods.

Keywords: hemolymph, antibacterial activity, clam*, R. philippinarum*

INTRODUCTION

Among the marine invertebrates, bivalve molluscs constitute the group of major economic importance. Oysters, mussels, scallops, cockles and various species of clams, worth billions of dollars, are harvested each year in various parts of the world (*FAO database*). Epizootics in commercially exploited species have repeatedly struck the related industries, causing great economic loss. One of the latest examples of these epizootics is the brown ring disease that appeared first in France in 1987 and spread throughout aquaculture sites for the manila clam *Ruditapes philippinarum* along the European Atlantic coasts (Allam, 1998). This disease is caused by a *Vibrio* strain first described as *Vibrio* P1 (Paillard & Maes, 1990 ; Paillard *et al.*, 1994) and named more recently *Vibrio tapetis* (Borrego *et al.*, 1996). Experimental induction of the disease has been demonstrated by inoculation of *V. tapetis* in the pallial cavity (Paillard *et al.*, 1994).

Against pathogens, bivalve molluscs are armed with an internal defense system which includes both cellular and humoral mechanisms (Chu, 1988, Feng, 1988). Hemocytes, the circulating blood cells, display several functions as phagocytosis, encapsulation, wound repair, and synthesis of humoral defense factors (see reviews by Cheng, 1996 and Feng, 1988). Diverse types of serum defense factors occur such as agglutinin/opsonins, bactericidins, antiparasitic factors and lytic enzymes (Chu, 1988; Millar & Ratcliffe, 1994).

The knowledge of antibacterial factors and their mode of induction are of interest in the case of bacterial diseases as the brown ring disease (BRD) in *R. philippinarum*. Previous work on this disease showed that experimental challenge of clams with the bacteria *V. tapetis* induced cellular responses in hemolymph, i.e. changes in hemocyte counts (Oubella *et al.*, 1996). The only humoral response demonstrated is a temporary rise in leucine amino-peptidase activity, an enzyme suspected to act in non-specific defense against microorganisms (Oubella *et al.*, 1994). This paper reports the induction of an antibacterial activity in the hemolymph of *R. philippinarum* following challenge with its pathogen *V. tapetis*, along with changes in hematological parameters. Hemocyte counts were performed to obtain information on cellular responses to infection in hemolymph and to possibly explain the origin of antibacterial factors.

MATERIAL AND METHODS

Animals

Healthy adult (30-35 mm) *R. philippinarum* were collected from a wild population in an estuary inside the Bay of Brest (France). The animals were maintained in marine aerated aquarium at 15°C and fed daily throughout experiments using cultured monocellular algae (*Dunaliella euchlora*, *Pavlova lutheri*, *Isochrysis galbana*).

Infection experiment

A suspension of *V. tapetis* ($5x10^8$ cfu ml^{-1}) in sterile seawater (SSW) was obtained from a 72 h culture (laboratory collection) grown on marine agar. Clams were challenged with *V. tapetis* according to two different protocols. A part of the clams (N=60) received 0.5 ml ($5x10^7$ cfu in SSW) of a diluted suspension into the pallial cavity. According to Paillard & Maes (1990), this way of challenge induces the BRD symptoms within days. A second protocol was designed to inoculate the pathogen into the body. The clams (N=60) received 0.1 ml ($5x10^7$ cfu in SSW) of the initial suspension into the posterior adductor muscle. Two series of control individuals (60 clams each) received sterile seawater, one in the pallial cavity, other in the muscle. Thereafter, the clams were maintained in four separated tanks. Ten clams were sampled from each tank at 4 hours, 1 day, 3 and 7 days (except for Day 1, N=12). Fifteen clams were processed before any treatment to have initial parameters.

Hemolymph sampling and defense parameters measurement

For each clam, about 400 µl of hemolymph was collected from the posterior adductor muscle sinus according to Auffret & Oubella (1995). Total hemocyte counts (THC) were immediately made in a Malassez hemocytometer. In order to obtain whole hemolymph extracts, a volume of about 300 µl was sonicated (40 min), filtered (0.2 µm), aliquoted in sterile Eppendorff vials and stored frozen (-20°C) until processing. Total protein contents of these extracts (serum and cell lysate) was determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

The antibacterial activity of hemolymph was measured using a plate test according to Xylander & Nevermann (1990). Standardised solid agar plates (15 ml of tryptone soya agar) were overlayed with a *Micrococcus luteus* suspension (approximately 10^7 cfu/plate). Holes of 6 mm in diameter were punched into the agar and received in duplicate 60 µl whole hemolymph extracts diluted to obtain a standard protein concentration of 50 µg ml^{-1} as recommended by Smith *et al.* (1995). After incubation (72 hr at 30°C), the antibacterial activity in a sample appeared as the development of a clear zone around the hole (free of visible bacterial colonies).

Its area was calculated ($S = \pi R^2 - \pi r^2$, S = area, R = radius of clear zone, r = radius of the hole = 3 mm) and expressed in square mm (Fig. 1). Parametric and non-parametric statistical tests were employed to analyse possible differences in values between test and control clams. To evaluate the contribution of hemolymph lysozyme in the overall antibacterial activity observed in control and inoculated clams, a standard curve of antibacterial activity (expressed in mm^2) was build using known concentrations of chicken egg-white lysozyme (CEWL).

Figure 1 : Antibacterial activity of the hemolymph was assessed by inhibition zone assay. The activity, here a clam inoculated whithin the adductor muscle with *V. tapetis*, was measured as the surface area of clear zone = $S = \pi R^2 - \pi r^2$ and expressed in mm^2.

RESULTS

Development of the brown ring syndrome

The BRD symptoms (conchiolin deposits on the inner face of the shell, Paillard *et al*., 1994) were monitored under a stereomicroscope to assess the development of the disease in all the experimental clams. Among challenged individuals, only those injected into the pallial cavity displayed the characteristic brown spots in 4 clams among 10 at day 3 and in 6 among 10 at day 7.

Antibacterial activity

Antibacterial activity against *M. luteus* was present in the hemolymph of untreated clams sampled at time 0 (Table 1). Similar values were measured until day 1 in both series of control clams which had received sterile seawater, either in the muscle or in the pallial cavity (Table 1). This could therefore be considered as a base line activity in hemolymph. An unexplained, slight decrease was however observed

in SSW-injected clams at day 3 and day 7. The levels rose in all challenged clams from day 3 to the end of the experiment. Inoculation in the muscle induced aearly response since the activity significantly increased at day 1 compared to the control ($p < 0.01$).

Table 1. Antibacterial activity (surface of clear zone in mm^2, mean ± standard error) at different time intervals following challenge with *Vibrio tapetis* (VT). Control clams were injected with sterile sea water (SSW).

		Time (days)			
Time 0	**Treatments**	4 hours	1	3	7
35 ± 5	**SSW-AM**	37 ± 4 [a]	28 ± 3 [a, b]	20 ± 4 [b, c]	14 ± 2 [c]
	VT-AM	55 ± 8 [a]	94 ± 10 ** [b]	127 ± 8 ** [c]	151 ± 13 ** [c]
	SSW-PC	31 ± 4 [a]	34 ± 4 [a]	11 ± 2 [b]	6 ± 2 [b]
	VT-PC	35 ± 6 [a]	37 ± 5 [a]	168 ± 9 ** [b]	174 ± 19 ** [b]

SSW-AM and VT-AM : clams injected within the posterior adductor muscle. SSW-PC and VT-PC : clams injected into the pallial cavity. N = 10, except for untreated clams (time 0, N15 =) and day 1 (N12 =). Significant differences between test clams and their respective controls were noted at the 0.05 level (*) and the 0.01 level (**) (Mann-Whitney test). a, b and c represent differences whithin each treated group (ANOVA, p = 0.05).

Hemocyte counts

Results of THC are given in Table 2. Clams inoculated into the muscle had significantly higher THC values than SSW-injected individuals at day 1 and day 3. The increase was weaker at day 7 ($p = 0.241$). Clams inoculated into the pallial cavity had a significantly higher THC at day 7 compared to their control ($p = 0.041$).

DISCUSSION

Results of the development of BRD few days following inoculation of *V. tapetis* into the pallial cavity are in accordance with previous work (Paillard *et al*., 1994; Allam *et al*., 1996). The fact that clams injected with *V. tapetis* in the muscle did not develop the symptoms within this delay may indicate that internal mechanisms had been efficient to inactivate this pathogen. However, this could also be the result of the failure of *V. tapetis* to colonize and disturb the periostracal

lamina. Indeed, BRD symptoms normally appear after *V. tapetis* contact with the periostracal lamina-mantle edge, which was artificially avoided here since bacteria were directly injected inside the muscle.

The increase in total hemocyte counts (THC) was considered as an hemocytosis by Oubella *et al.* (1994, 1996) in similar conditions. Moreover, we demonstrate here that the inoculation into the muscle also induced an hemocytosis, in agreement with observations by Suresh & Mohandas (1990) in other clam species. In molluscan hemolymph, hemocytosis is one of the major cellular responses of the defense system (Feng, 1988; Cheng, 1996). The role of bacterial factors are therefore to be considered to explain the induction of the cellular response to the BRD agent.

Table 2. Total hemocyte counts (10^6 cells ml^{-1}, mean ± standard error) at different time intervals following challenge with *Vibrio tapetis* (VT). Control clams were injected with sterile sea water (SSW).

		Time (days)			
Time 0	**Treatments**	4 hours	1	3	7
2.3 ± 0.3	**SSW-AM**	2.5 ± 0.3	2.8 ± 0.2	2.6 ± 0.2	2.9 ± 0.3
	VT-AM	2.9 ± 0.6	4.1 ± 0.5 **	4.4 ± 0.6 *	3.8 ± 0.5
	SSW-PC	NA	NA	NA	2.6 ± 0.3
	VT-PC	NA	NA	NA	4.1 ± 0.6 *

SSW-AM and VT-AM: clams injected within the posterior adductor muscle. SSW-PC and VT-PC : clams injected into the pallial cavity. NA: not assayed. N = 10, except for untreated clams (time 0, N15 =) and day 1 (N12 =). Significant differences between test clams and their respective controls were noted at the 0.05 level (*) and the 0.01 level (**) (Mann-Whitney test). No differences were detected whithin treatments (ANOVA, p > 0.05).

Previous studies reported the occurrence and induction of a bactericidal activity in oysters *Crassostrea gigas* and *Ostrea edulis* after challenge with various environmental bacterial strains (Mori, 1985, Hubert *et al.*, 1996 a and b). However, we demonstrate here, by using a pathogen responsible for a disease in *R. philippinarum*, that internal defense is enhanced by experimental challenge. Whether or not the observed antibacterial activity is cell- or serum-mediated is not known since whole hemolymph extracts were used. A cellular origin is suspected since Hubert *et al*. (1996 b) have found in oysters that the major part of the

antibacterial activity was in the cell fraction. Thus, the increase in antibacterial activity is likely to be related to the observed hemocytosis. However, all the phenomena described cannot be explained quantitatively by changes in cell number. Specially, in clams injected in the adductor muscle, there was a shift between the rise of THC which was near the maximum from day 1, and the regular increase of antibacterial activity from day 1 to day 7. The second argument is that the heavy changes occurred in the antibacterial activity to THC ratio suggesting the induction of secretion by the cells. To address these questions, changes in the specific antibacterial activity in the serum fraction (activity vs total protein) or in the cell fraction (activity vs cell number) should be investigated.

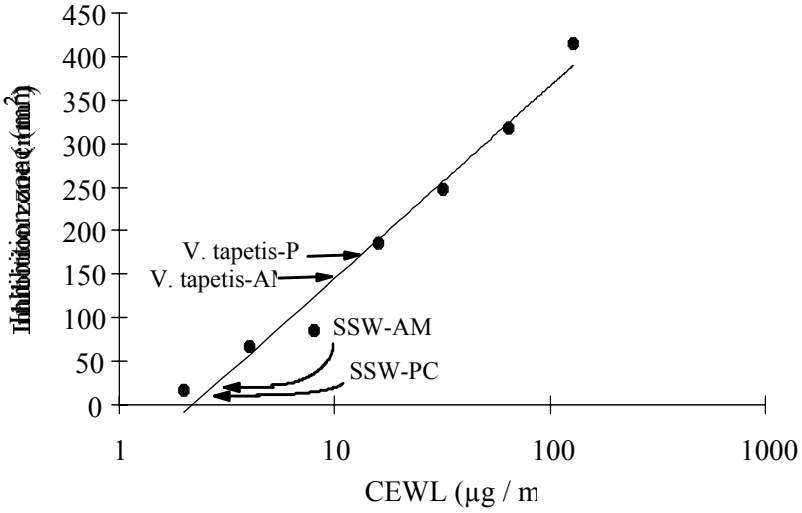

Figure 2 : Antibacterial activity of chicken egg-white lysozyme (filled circles). Mean activity of each experimental clam batch at day 7 is indicated. Hemolymph extracts were previously diluted to obtain a standard protein concentration of 50 µg ml^{-1}.

Several antibacterial factors have been described in bivalve molluscs, including lysozyme and peptides. To evaluate the contribution of lysozyme in the overall antibacterial activity observed, we used a standard curve established from chicken egg-white lysozyme (CEWL, Figure 2). At day 7, the antibacterial activity in clams inoculated with *V. tapetis* ranged from 10.6 to 13.5 µg CEWL equivalent per ml (212 to 270 µg CEWL equivalent per mg proteins) (Figure 2). These values are 10 to 30 fold higher than values of lysozyme contents determined by turbidometric methods using lyophilised *M. luteus* in *R. philippinarum* (Allam & Paillard, 1998), *R. decussatus* (Lopez-Gomez, 1995) and other marine bivalve

species (Santarem *et al*., 1994; Chu & La Peyre, 1993). The high antibacterial activity measured here against live *M. luteus* suggests the involvement of other antibacterial factors than lysozyme as antibacterial peptides. Inducible antibacterial peptides as defensin have been recently reported in the hemolymph of the edible mussel *Mytilus edulis*, and are suspected to occur in *C. gigas* and *O. edulis* (Hubert *et al*., 1996 a and b). Such antibacterial peptides are of a major interest in cultured bivalves, particularly *R. philippinarum*, since they could be used in attempts to increase resistance to bacterial diseases by genetic manipulation.

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