Aquat. Living Resour., 1996, **9**, 137-143

Selective induction of hemocytic response in *Ruditapes philippinarum* (Bivalvia) by different species of *Vibrio* (Bacteria)

Radouane Oubella, Philippe Maes, Bassem Allam, Christine Paillard and Michel Auffret

Laboratoire « Flux de matière et réponse du vivant », URA CNRS D1513, Institut Universitaire Européen de la Mer, Université de Bretagne Occidentale, B.P. 809, 29285 Brest Cedex, France.

Received June 15, 1995; accepted January 11, 1996.

Oubella R., P. Maes, B. Allam, C. Paillard, M. Auffret. *Aquat. Living Resour.*, 1996, **9**, 137-143.

Abstract

Adult, healthy Manila clams, *Ruditapes philippinarum*, were inoculated in the laboratory with different, possibly pathogenic bacteria species to study the cellular immune responses. Challenging the clams with *Vibrio* P1, the causative agent of brown ring disease, induced significant increased total hemocyte counts three days post-challenge. The triggering role of the number of bacteria in the inoculum suggested a threshold for inducing the host response. In addition, a significant decrease of hyalinocytes and an increase of granulocytes revealed a modification in hemocyte population balance. No response was observed in individuals that received either heat killed-*Vibrio* P1, *V. anguillarum* or *V. pelagius*. These experiments demonstrate in *R. philippinarum* the influence of bacterial parameters on the selective induction of host immunodefence mechanisms. The biological processes by which intra-pallial inoculation of *Vibrio* P1 stimulates the internal defence system of the host has not yet been identified but the role of some bacterial factors (toxins, adherence capacity) is discussed.

Keywords: Hemocytic responses, immunodefence, bacterial challenge, *Vibrio, Ruditapes philippinarum.*

Induction sélective de la réponse hémocytaire chez Ruditapes philippinarum *(Bivalvia) par différentes espèces de* Vibrio *(Bacteria)*

Résumé

Des palourdes japonaises, *Ruditapes philippinarum*, adultes et saines, ont été inoculées au laboratoire par différentes espèces de bactéries potentiellement pathogènes pour étudier les réponses immunitaires à médiation cellulaire. L'inoculation de palourdes avec *Vibrio* P1, agent responsable de la maladie de l'anneau brun, induit une augmentation significative de la concentration en hémocytes circulants après trois jours de mise en contact. Le rôle déterminant du nombre de bactéries dans l'inoculum suggère la présence d'un seuil dans l'induction de la réponse chez l'hôte. Une diminution significative des hyalinocytes et l'augmentation des granulocytes révèle en outre un déséquilibre dans les populations hémocytaires. Aucune réponse n'a été observée chez les individus inoculés soit avec *Vibrio* P1 tué à la chaleur, soit avec *V. pelagius* ou *V. anguillarum*. Ces expériences démontrent l'influence des paramètres bactériens sur l'induction sélective des mécanismes d'immunodéfense de *R. philippinarum*. Les processus biologiques impliqués dans la stimulation du système de défense interne après inoculation intra-palléale de *Vibrio* P1 ne sont pas encore identifiés, mais le rôle de certains facteurs bactériens (toxines, capacité d'adhérence) est discuté.

Mots-clés : Réponses hémocytaires, immunodéfense, inoculation bactérienne, *Vibrio, Ruditapes philippinarum.*

INTRODUCTION

The survival and development of marine invertebrate populations in their environment rely in part on the efficiency of their internal defence system (IDS). In bivalve molluscs, the cellular mechanisms, mainly phagocytosis and encapsulation, allow clearance of invading microorganisms, including pathogenic bacteria, from internal compartments (Rodrick and Ulrich, 1984; Fisher, 1986; Feng, 1988). In several molluscs, early signs of IDS involvement after challenging with foreign microorganisms have been reported as elevated total hemocyte counts (THC) (Suresh and Mohandas, 1990; Mounkassa and Jourdane, 1990) and modifications in differential hemocyte counts (DHC) (Foley and Cheng, 1974; Ford *et al.*, 1993). In the Manila clam, *Ruditapes philippinarum*, previous experiments have shown that challenge with the bacterium *Vibrio* P1, causative agent of brown ring disease (BRD), resulted in elevated THC within days post intra-pallial inoculation (Oubella *et al.*, 1993, 1994). This hemocytosis was relevant to the stimulation of antibacterial defence mechanisms in these organisms.

The quality of the response of clam IDS during the period following the challenge is certainly a major factor for the success or failure of the infective process. Especially, the role of the pathogenicity of the bioagressor, its virulence and the interactions with the host have to be considered. Experimental infections of several bivalve species with *Vibrio* P1, showed a high sensitivity only in *R. philippinarum* which developed the symptoms of BRD (Maes, 1992; Maes and Paillard, 1992; Paillard *et al.*, 1994). In addition, challenging *R. philippinarum* with different bacteria strains revealed that only *Vibrio* P1 induced the disease (Maes, 1992; Maes and Paillard, 1992). These observations suggested some specificity of the pathogen towards the host. On the way to understanding defence mechanisms against bacterial diseases, investigations on cellular responses of *R. philippinarum* IDS after various bacterial challenges were thought to provide interesting data on early phenomena, even in case of failure of the disease process.

In this contribution, adult Manila clams *R. philippinarum* were inoculated with either live or killed *Vibrio* P1, *V. anguillarum*, a pathogen for marine fish and for shellfish larvae (Myhr *et al.*, 1991; Riquelme *et al.*, 1995), or *V. pelagius*, non-pathogenic vibrion frequently detected in seawater and healthy clams (Castro *et al.*, 1992). The experimental protocol was based on a short-term challenge previously developed in our laboratory (Oubella *et al.*, 1993). In addition to the measurement of cytometric parameters (THC and DHC), immunological detection of *Vibrio* P1 was applied to tissue sections. In a complementary experiment, clams were inoculated with different doses of live *Vibrio* P1.

MATERIAL AND METHODS

Animals handling

Adult clams, *Ruditapes philippinarum*, were obtained in March 1992 from the Bay of Arcachon (France). To avoid inter-individual variation (Auffret and Oubella, 1994) animals were approximately the same age and size (shell length = 35.9 mm; SE = 0.5; N = 300). The clams did not display any symptom of BRD. They were acclimated for two weeks in the laboratory prior to challenge experiments. They were maintained in tanks with aerated seawater (15 ± 1°C and 30 ± 1‰) and fed daily with cultured algae (*Isochrysis galbana* and *Dunaliella euchlora*). Similar conditions were applied after inoculation.

Strains and inocula

The *Vibrio* P1 strain was isolated from diseased *R. philippinarum* from the Bay of Brest (Brittany, France) according to the method of Maes (1992). Killed *Vibrio* P1 was obtained by treatment with heat (60°C, 2 h). The marine pathogen, *Vibrio anguillarum*, was isolated from diseased fishes. *Vibrio pelagius* (ATCC 25916) was obtained from ambient seawater. All bacteria strains were cultured for 72 h on marine agar at 20°C prior to challenge experiments.

To test bacterial strains, four groups of 50 clams were inoculated separately with a suspension optically adjusted in sterile seawater to a concentration of about 10^8 cfu (colony forming unit) per ml. A volume of 0.5 ml of the suspension (about 5×10^7 bacteria) was injected into the pallial cavity of each individual. Control clams were injected with 0.5 ml sterile seawater. After inoculation, the clams were left out of water for 3 h to ensure retention of the bacteria in the pallial cavity, and then placed in separate tanks. To test the effect of the number of bacteria, 60 clams were inoculated with doses of live *Vibrio* P1 ranging from 5×10^2 to 5×10^6 bacteria per individual. Control clams were injected with sterile seawater.

Clam sampling

Both experiments were continued over a period of three days. In the first experiment, a preliminary sample of 20 clams was analyzed before the bacterial challenge (day 0). Thereafter, 20 clams from each of the five conditions were sampled at day 1 and day 3. In the second experiment, all the clams were analyzed after 3 days.

Hemolymph sampling and analysis of cytometric parameters

Hemolymph was collected from each clam ($n = 20$ per sample) with a hypodermic needle from the posterior adductor muscle sinus as previously described (Auffret and Oubella, 1995). THC was

measured with a Malassez hemocytometer after fixation of hemocytes (1:1) in an isoosmotic fixative solution. The fixative was 3% glutaraldehyde in 0.1 M cacodylate buffer (pH = 7.4) to which saccharose has been added (final osmolarity = 1 100 mOsmol.kg^{-1}) (Auffret and Oubella, 1995).

To determine DHC from *Vibrio* P1-challenged clams, hemocyte monolayers were processed by collecting hemolymph into a syringe containing hemocyte anti-aggregate solution (1:2) which was 1.5% EDTA in 0.1 M phosphate buffer (pH = 7.5) supplemented with 2% NaCl (Auffret and Oubella, 1995). The hemocyte suspension was adjusted with Malassez hemocytometer to 200-300 cells per mm^3. The cells were allowed to settle on a glass slide in a cytocentrifuge (30 g, 10 min). Prior to cytological examination, monolayers were stained by using a modified Pappenheim's technique (Auffret, 1989). DHC was determined from 250 hemocytes in each monolayer ($n = 5$ individuals per sample).

Histological and immunological techniques

To localize *Vibrio* P1 in tissues of inoculated clams, a rabbit serum directed against *Vibrio* P1 was kindly supplied by Pr A. Dodin (Institut Pasteur, France). Challenged animals were fixed within their shells in aqueous Bouin's solution for 48 h. The shells were than carefully removed under a binocular microscope to preserve the structural integrity of the periostracal lamina. The tissues were dehydrated in graded ethanol and xylene series, then embedded in paraffin. Paraffin sections (5 μm thick) were cut for immunofluorescence labelling. Rehydrated tissue sections were pre-incubated in phosphate buffered saline (PBS) (0.1 M, pH 7.4). After pre-treatment in 20% normal-goat serum, the primary antiserum diluted 1:200 was applied to the tissue sections in moist chamber for 1 h at 30°C. A FITC goat anti-rabbit serum was then applied for 45 min at 30°C in the dark. The slides were then rinsed three times with PBS and mounted for epifluorescence microscopic examination at 450-490 nm. In parallel, tissue sections of clams not inoculated with *Vibrio* P1 were subjected to the same treatments to constitute controls.

Statistical analyses

In the first experiment, the mean values of THC in treatments (different bacteria species) were compared to control using Student's t-test. The data were initially tested for normality using Kolmogrov-Smirnov test. In the second experiment, the mean values of THC in clams inoculated with different bacteria doses were compared to control using one-way analysis of variance (ANOVA). For DHC, difference between the main hemocyte populations was assessed using an unpaired test (Mann-Whitney test).

RESULTS

Total hemocyte count (THC)

Following the challenge of *R. philippinarum* with live *Vibrio* P1 (5×10^7 bacteria), the THC increased significantly ($p < 0.01$) at three days. This response was not observed in clams challenged with either *V. pelagius*, *V. anguillarum* or heat-killed *Vibrio* P1 (*table* 1).

In the dose-effect experiment with live *Vibrio* P1, a marked increase ($p < 0.01$) after 3 days in THC occurred in clams inoculated with a suspension of 5×10^6 bacteria. Doses ranging from 5×10^2 to 5×10^4, had no effect compared to seawater-injected clams (*table* 2).

Cytology of *R. philippinarum* hemocytes

Circulating hemocytes were classified on the basis of size, staining reaction, and presence or absence of cytoplasm granules (Auffret and Oubella, 1995). The cells were divided into five groups: hyalinocytes, neutrophilic granulocytes, small basophilic hemocytes, multinucleated cells and particle-loaded cells. Identification of hyalinocytes relied upon: 1) the lack of cytoplasm granules and 2) a large ovoid or kidney-shaped nucleus with stippled chromatin and one or two nucleoli. The nucleus was most of the time located in the centre of the cell. The cytoplasm was light pink or grey in the large cells and blue in the smaller cells. The small basophilic hemocytes were generally spherical with a narrow ring of dark blue cytoplasm around a large round nucleus. The neutrophilic granulocytes

Table 1. – Mean total hemocyte count (THC, cell.mm^{-3}) in *Ruditapes philippinarum* inoculated with different *Vibrio* species. Three days post-challenge, a significant increase in THC was observed only in *Vibrio* P1 challenged-clams. The values within parentheses are standard error of the mean ($n = 20$ per sample). *: different value (*t*-test, $p < 0.01$) from the control (sterile seawater).

Time (days)	Total hemocyte count				
	Control	*Vibrio* P1	Heat-killed *Vibrio* P1	*Vibrio pelagius*	*Vibrio anguillarum*
0			2 497 (83)		
1	2 489 (116)	2 599 (95)	2 523 (111)	2 516 (93)	2 716 (174)
3	2 543 (102)	3 085 (117)*	2 547 (98)	2 589 (115)	2 765 (222)

Table 2. – Mean total hemocyte count (THC, cell.mm^{-3}) after three days in *Ruditapes philippinarum* inoculated with different doses of live *Vibrio* P1. Only the inoculum containing 5×10^6 bacteria elicited a significant response. Values in parentheses are standard error of the mean ($n = 20$ per sample). *: different value (ANOVA, $p < 0.01$) from the control (sterile seawater).

Inoculated fraction	Total hemocyte count
Control	2 608 (83)
5×10^2 bacteria	2 488 (86)
5×10^4 bacteria	2 596 (84)
5×10^6 bacteria	2 980 (97)*

contained shades of pink cytoplasm granules; they were characterized by an eccentric hyperchromatic nucleus. The multinucleated cells, were larger and exhibited two or more nuclei in their cytoplasm. The particle-loaded cells were characterized by various inclusion bodies in their cytoplasm, possibly reflecting their phagocytic activity.

Differential hemocyte count (DHC)

In control clams, both of the hyalinocytes and neutrophilic granulocytes populations contributed with a ratio of respectively 55 and 39% of total circulating hemocytes. Minor types were small basophilic hemocytes, multinucleated cells, and particle-loaded cells (*fig.* 1). In *Vibrio* P1 challenged-clams, the balance between the main hemocyte populations was modified. When compared to day 0, inoculation of live *Vibrio* P1 resulted after 3 days in a significant ($p < 0.05$) decrease of hyalinocyte number and a concomitant, non significant ($p = 0.14$), increase of neutrophilic granulocyte (*fig.* 2). No changes in the percentage of minor hemocyte types was observed in these clams.

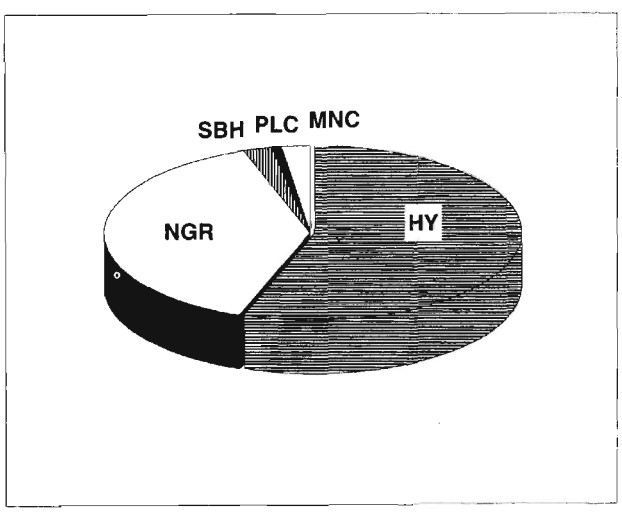

Figure 1. – Circulating hemocyte population proportions in adult *Ruditapes philippinarum*. HY and NGR constitute the main types of hemocytes. HY = hyalinocytes, NGR = neutrophilic granulocytes, SBH = small basophilic granulocytes, MNC = multinucleated cells, PLC = particle-loaded cells.

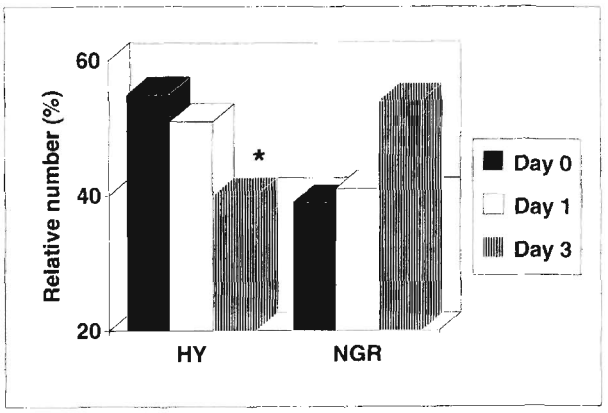

Figure 2. – Changes in the main hemocyte populations in *Ruditapes philippinarum* challenged with the pathogenic bacterium *Vibrio* P1. Three days post-inoculation resulted in a significantly decreased HY proportion and a concomitant increase of circulating NGR. *: different value (Mann-Whitney test, $p < 0.05$) from the control (sterile seawater). HY = hyalinocytes, NGR = neutrophilic granulocyte.

Detection of *Vibrio* P1 in tissues

In the immunofluorescence study, *Vibrio* P1 was observed adhering to the periostracal lamina of challenged *R. philippinarum* one (*fig.* 3A) and three (*fig.* 3B) days post-inoculation. No bacteria were observed in sections from control clams.

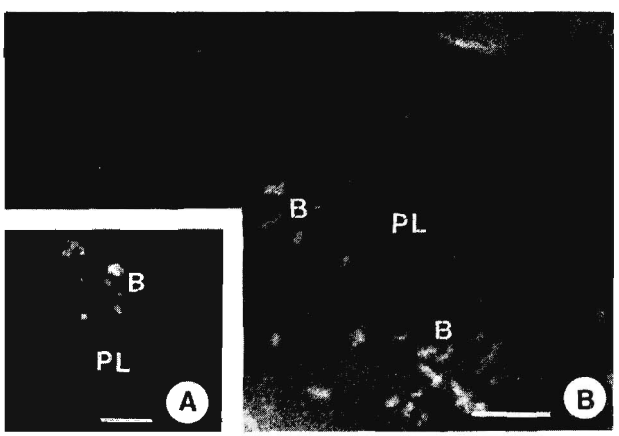

Figure 3. – Microscopic detection of VP1 in *Ruditapes philippinarum* (indirect immunofluorescence labelling). Adherence of *Vibrio* P1 to the periostracal lamina occurred one (A) and three (B) days post-challenge. PL = periostracal lamina, B = fluorescent bacteria. Scale bar = 10 μm.

DISCUSSION

In the present study, a cellular response to bacterial challenge was observed only in the case of live

Vibrio P1, through a conspicuous hemocytosis in the circulatory compartment of the clams. In accordance with previous work on bacterial infection in this clam species, this phenomenon was significant 3 days post-challenge, even if an increase would be observed after 24 h (Oubella *et al.*, 1993). Oubella *et al.* (1993, 1994) proposed that the elevated number of circulating cells resulted from a mobilization and consecutive migration of resident hemocytes from tissues towards the hemolymph compartment in response to host-pathogen interactions. A comparable explanation had been put forward in other cases of parasitic attack in various molluscs (Mounkassa and Jourdane, 1990; Ford *et al.*, 1993). The hypothesis of a migration was supported in part by the structure of the circulatory compartment in these invertebrates, which is an open system. In view of all these studies reporting the observation of comparable responses after challenging different species with different strains, the hemocytosis might be interpreted as a non-specific immune response of the host (*see* Feng, 1988).

In *Vibrio* P1-challenged clams, the induction of a hemocytosis was dependent on the number of inoculated bacteria. An inoculum containing a minimum of 5×10^6 bacteria was necessary to induce a significant hemocytic response. In these experimental conditions, this dose corresponded to a threshold for inducing the continuation of the infectious process and consequently, the host response.

In contrast, total hemocyte counts remained unchanged in clams receiving either heat-killed *Vibrio* P1, *V. pelagius* or *V. anguillarum*. This response demonstrates that some events following the entry of bacteria into the pallial cavity of the mollusc may vary depending on the strain and on the host species, leading or not to the disease process. In the case of *Vibrio* P1, killing by heat obviously altered its pathogenicity. Concerning the other strains, different explanations could be involved, including the ability for the immunodefence system to produce suitable defensive processes. However, virulent bacteria induce diseases even in immunocompetent organisms. In the case of adult clams, *V. pelagius* or *V. anguillarum* might be rather considered as opportunistic bacteria. This is sustained by previous observations that no macroscopical signs of disease arose after inoculation of these strains (Maes, 1992; Maes and Paillard, 1992). Concerning *V. pelagius* which may occur in the environment of the clams and *V. anguillarum* which is pathogenic only towards larval molluscs, epithelial borders of the pallial cavity would be potential efficient barriers able to stop or eliminate some microorganisms.

Concerning live *Vibrio* P1, the modalities for the induction of the immune response are not yet known. According to a general scheme of the leucocytic response and recruitment reviewed by Gallin (1980), stimuli would originate at sites of host to pathogen contact or at sites of bacterial entry in tissues. In vertebrates, the molecular signals between stimuli and host responses are cell factors (*e.g.* enzymes) and bacterial factors (*e.g.* toxins, wall components). Migration of mammalian immunocompetent cells by way diapedesis occurs through chemotaxis toward substances released by infecting microorganisms (Marasco and Ward, 1988; Beeken *et al.*, 1995). In clams challenged with live *Vibrio* P1, the observed hemocytosis could occur through stimuli produced by bacteria adhering to tissues, producing irritation and even entering the host. Indeed, bacterial bodies have been observed by microscopy in the digestive gland of *Vibrio* P1-infected clams (Plana and Le Pennec, 1991). In other bivalves, chemotactic migration of hemocytes mediated by secreted bacterial products has been demonstrated *in vitro* in the case of *C. virginica* (Cheng and Howland, 1979; Howland and Cheng, 1982), *Mytilus edulis* (Schneeweiβ and Renwrantz, 1993), and the clam *Mercenaria mercenaria* (Fawcett and Tripp, 1994). An interesting finding is that hemocytes did migrate to living, but not to killed bacteria (Cheng and Howland, 1979; Fawcett and Tripp, 1994). Borrego *et al.* (1996) found that *Vibrio* P1 strains are able to synthesize and produce toxins. These xenobiotics could be recognized as nonself and act as stimuli for hemocyte mobilization into the circulatory compartment.

Whether the observed changes in hemolymph were a direct consequence of the bacteria-generated pathogenic processes or a physiological response by the host has to be determined. This question is suggested by experiments in the gastropod mollusc *Biomphalaria glabrata*, where physico-chemical factors were involved in changes of total hemocyte counts (Wolmarans and Yssel, 1988). However, it is likely that bacterial factors are much involved. The pathogenicity of *Vibrio* P1 towards *R. philippinarum* has been showed by experimental inoculations of various *Vibrionaceae* species isolated from diseased individuals where the development of macroscopical symptoms of the disease occurred only in *Vibrio* P1-challenged clams (Maes, 1992; Paillard *et al.*, 1994).

Microbial adherence and pathogenicity have been correlated in many organisms, including mammals (Yamamoto and Yokota, 1989; Lee *et al.*, 1989), fish (Chen and Hanna, 1992; Esteve *et al.*, 1993), crustaceans (Chen and Hanna, 1994) and bivalve molluscs (Riquelme *et al.*, 1995). Recently, morphological characteristics possibly related to virulence have been identified in *Vibrio* P1 infecting *R. philippinarum* (Paillard *et al.*, 1994; Borrego *et al.*, 1996; T. H. Birkbeck, unpublished data). Attachment of this bacteria to host structures appeared to be mediated by filamentous appendages characterized as pili, a mechanism considered to be an important step in host colonization (Nakason and Iwanaga, 1990). In this study, *Vibrio* P1 immunolabelling in tissues revealed attachment to the surface of the periostracal lamina 3 days post-challenge, suggesting

a relationship between its virulence and its capacity to adhere to clam tissues. This finding confirms the recent observations in the same species of early attachment 12 h post-inoculation (Paillard and Maes, 1995*a, b*). In other cases of vibriosis in marine bivalves, attachment of bacteria to the outer surfaces preceded subsequent invasion of the mantle and soft tissues (Elston and Leibovitz, 1980; McHenry and Birkbeck, 1986) leading to tissue damage (Nottage and Birkbeck, 1986). Attachment to host tissues depends on bacteria viability. Loss of adherence by killed *Vibrio* was reported by Iijima *et al.* (1981) in mammals. This could explain the observation that heat-killed *Vibrio* P1 did not induce the hemocytic response.

Two major hemocyte populations occur in the hemolymph of *R. philippinarum*: the hyalinocytes and the neutrophilic granulocytes, the former type being the most abundant (about 60%) in healthy specimens (Auffret and Oubella, 1994). In *Vibrio* P1-challenged *R. philippinarum*, the balance between these populations was affected, with a significant decrease of hyalinocytes and an increase of granulocytes. The other minor hemocyte types (small basophilic hemocytes, multinucleated cells and particle-loaded cells) were not involved. This conspicuous inversion might reflect the greater involvement of one of these types in antibacterial defence. Histological examinations showed tissue-infiltrating hemocytes in *Vibrio* P1-infected *R. philippinarum* (Santamaria *et al.*, 1995). However, selective cytotoxic effects of bacterial toxins could have comparable effects and even enhance it. Pathogenic *Vibrio* strains are able to produce hemolysins (Kumazawa *et al.*, 1985). According to Nottage and Birkbeck (1990), cultured *Vibrio* strains secreted substances with *in vitro* cytotoxic effects against bivalve hemocytes. Recent, unpublished findings by these authors have confirmed the cytotoxicity of *Vibrio* P1 to *R. philippinarum* hemocytes. It couls explain in part the observed changes in hemogram composition.

CONCLUSION

Thus study confirms that in bivalve molluscs, hemocytosis is a true immune response. In addition, variations in the balance between cell types are in agreement with functional differences among hemocyte populations. The demonstration of a selective induction of the hemocytic response depending on bacterial parameters is made in *R. philippinarum*. Clams inoculated with live *Vibrio* P1 displayed a hemocytosis when no response was observed in individuals that received heat killed-*Vibrio* P1, *V. anguillarum* or *V. pelagius*. In addition, a sufficient number of bacteria challenging the clams was necessary to induce the response. The mechanisms by which intra-pallial inoculation of *Vibrio* P1 stimulate the internal defence system has not yet been identified and defined, although it is suggested that bacterial products such as toxins play a role. Such short-term experiments designed from this novel host-pathogen model appear to be a tool to investigate the interactions between different strains or ecotypes of pathogenic bacteria and the internal defence system of bivalve molluscs.

Acknowledgements

The authors would like to thank Dr M. Johnson for reviewing and correcting this paper and A. Marhic and A. Le Mercier for their technical assistance. Many thanks to Dr M. Vigneulles (Fish Pathology Laboratory, CNEVA Brest) for supplying *Vibrio anguillarum* strain. Research supported by the Commission of the European Communities (FAR Programme, contract number AQ-3-367). This paper is contribution n° 95-006 for URA CNRS D 1513.

REFERENCES

Auffret M. 1989. Comparative study of the hemocytes of two oyster species: the European flat oyster, *Ostrea edulis* Linnaeus, 1750 and the Pacific oyster, *Crassostrea gigas* (Thunberg, 1793). *J. Shellfish Res.* **8,** 367-373.

Auffret M., R. Oubella 1994. Cytometric parameters of bivalve molluscs: effect of environmental factors. *In*: Modulators of Fish Immune Responses, Stolen J. S., T. C. Fletcher eds. SOS Publications, Fair Haven U.S.A. **1,** 23-32.

Auffret M., R. Oubella 1995. Cytological and cytometric analysis of bivalve mollusc hemocytes. *In*: Techniques in Fish Immunology, Stolen J. S., T. C. Fletcher, S. A. Smith, J. T. Zelikoff, S. L. Kaattari, R. S. Anderson, K.Söderhäll, B. A. Weeks-Perkins eds. SOS Publications, Fair Haven U.S.A. **4,** 55-64.

Beeken W., J. Fabian, J. Fenwick 1995. The chemotactic response of blood neutrophils and monocytes to strains of *Escherichia coli* with different virulence characteristics. *J. Med. Microbiol.* **40,** 196-199.

Borrego J. J., A. Luque, D. Castro, J. A. Santamaria, E. Martinez-Manzanares 1996. Virulence factors of *Vibrio* P1, the causative agent of brown ring disease in the Manila clam, *Ruditapes philippinarum*. *Aquat. Living Resour.* **9,** 125-136.

Castro D. E., Martinez-Manzanares, A. Luque, B. Fouz, M. A. Morinigo, J. J. Borrego, A. E. Toranzo 1992.

Characterization of strains related to brown ring disease outbreaks in southwestern Spain. *Dis. Aquat. Org.* **14,** 229-236.

Chen D., P. J. Hanna 1992. Attachment of *Vibrio* pathogens to cells of *Oncorhynchus mykiss* (Richardson), the rainbow trout. *J. Fish. Dis.* **15,** 371-377.

Chen D., P. J. Hanna 1994. Immunodetection of specific *Vibrio* bacteria attaching to tissues of the giant tiger prawn *Penaeus monodon. Dis. Aquat. Org.* **20,** 159-162.

Cheng T. C., K. H. Howland 1979. Chemotactic attraction between hemocytes of the oyster, *Crassostrea virginica* and bacteria. *J. Invertebr. Pathol.* **33,** 204-210.

Elston R., L. Leibowitz 1980. Pathogenesis of experimental vibriosis in larval American oyster, *Crassostrea virginica. Can. J. Fish. Aquat. Sci.* **37,** 964-978.

Esteve C., G. Hayashida, E. G. Biosca, C. Amaro 1993. Virulence of *Aeromonas hydrophila* and some other bacteria isolated from European eels *Anguilla anguilla* reared in fresh water. *Dis. Aquat. Org.* **16,** 15-20.

Fawcett L. B., M. R. Tripp 1994. Chemotaxis of *Mercenaria mercenaria* hemocytes to Bacteria *in vitro. J. Invertebr. Pathol.* **63,** 275-284.

Feng S. Y. 1988. Cellular defence mechanisms of oysters and mussels. *Am. Fish. Soc. Spec. Publ.* **18,** 153-168.

Fisher W. S. 1986. Structure and function of oyster hemocytes. *In*: Immunity in invertebrates, M. Brehelin ed. Springer-Verlag, Berlin, 25-35.

Foley D. A., T. C. Cheng 1974. Morphology, hematologic parameters, and behaviours of hemolymph cells of the quahog clam, *Mercenaria mercenaria. Biol. Bull.* **146,** 343-356.

Ford S. E., S. A. Kanaley, D. T. J. Littlewood 1993. Cellular responses of oysters infected with *Haplosporidium nelsoni*: changes in circulating and tissue-infiltrating hemocytes. *J. Invertebr. Pathol.* **61,** 49-57.

Gallin J. I. 1980. The cell biology of leukocyte chemotaxis. *In*: The cell biology of inflammation, Glynn L. E., J. C. Houch, G. Weissmann eds. Elsevier, 299-335.

Howland K. H., T. C. Cheng 1982. Identification of bacterial chemoattractants for oyster (*Crassostrea virginica*) hemocytes. *J. Invertebr. Pathol.* **39,** 123-132.

Iijima Y., H. Yamada, S. Shinoda 1981. Adherence of *Vibrio parahaemolyticus* and its relation to pathogenicity. *Can. J. Microbiol.* **27,** 1252-1259.

Kumazawa N. H., E. Kato, Y. Nakagawa 1985. Preliminary analyses on persistence of *Vibrio Parahaemolyticus* in a Brackish water clam, *Corbicula japonica. Jpn. J. Vet. Sci.* **48,** 267-271.

Lee K. K., P. Doig, R. T. Irvin, W. Paranchym, R. S. Hodges 1989. Mapping the surface regions of *Pseudomonas aeruginosa* PAK pili: the importance of the C-terminal region for adherence to human buccal epithelial cell. *Mol. Microbiol.* **3,** 1493-1499.

McHenry J. G., T. H. Birkbeck 1986. Inhibition of filtration in *Mytilus edulis* by marine vibrios. *J. Fish. Dis.* **9,** 257-261.

Maes P. 1992. Pathologie bactérienne chez deux invertébrés marins d'intérêt commercial, *Ruditapes philippinarum* et *Paracentrotus lividus*. Thèse dr. Bretagne occidentale, Brest, 217 p.

Maes P., C. Paillard 1992. Effet de *Vibrio* P1, pathogène de *Ruditapes philippinarum* sur d'autres espèces de bivalves. Haliotis **14,** 141-148.

Marasco W. A., P. A. Ward 1988. Chemotactic factors of bacterial origin. *Methods Enzymol.* **162,** 198-214.

Mounkassa J. B., J. Jourdane 1990. Dynamics of the leukocytic response of *Biomphalaria glabrata* during the larval development of *Schistosoma mansoni* and *Echinostoma liei. J. Invertebr. Pathol.* **55,** 306-311.

Myhr E., J. L. Larsen, A. Lillehaug, R. Gudding, M. Heum, T. Hastein 1991. Characterization of *Vibrio anguillarum* and closely related species isolated from farmed fish in Norway. *Appl. Environ. Microbiol.* **57,** 2750-2757.

Nakason N., M. Iwanaga 1990. Pili of a *Vibrio parahaemolyticus* strain as a possible colonization factor. *Infect. Immunol.* **58,** 61-69.

Nottage A. S., T. H. Birkbeck 1986. Toxicity to marine bivalves of culture supernatant fluids of the bivalve-pathogenic *Vibrio* strain NCMB 1338 and other marine vibrios. *J. Invertebr. Pathol.* **9,** 249-256.

Nottage A. S., T. H. Birbeck 1990. Interactions between different strains of *Vibrio alginolyticus* and hemolymph fractions from adult *Mytilus edulis. J. Intertebr. Pathol.* **56,** 15-19.

Oubella R., P. Maes, C. Paillard, M. Auffret 1993. Experimentally induced variation in hemocyte density for *Ruditapes philippinarum* and *Ruditapes decussatus* (Mollusca, Bivalvia). *Dis. Aquat. Org.* **15,** 193-197.

Oubella R., C. Paillard, P. Maes, M. Auffret 1994. Changes in hemolymph parameters in the manila clam *Ruditapes philippinarum* (Mollusca, Bivalvia) following bacterial challenge. *J. Invertebr. Pathol.* **64,** 33-38.

Paillard C., P. Maes, R. Oubella 1994. Brown ring disease in clams. *Ann. Rev. Fish. Dis.* **4,** 219-240.

Paillard C., P. Maes 1995*a*. The brown ring disease in the Manila clam, *Ruditapes philippinarum*. 1: Ultrastructural alterations of the periostracal lamina. *J. Invertebr. Pathol.* **65,** 91-100.

Paillard C., P. Maes 1995*b*. The brown ring disease in the Manila clam, *Ruditapes philippinarum*. 2: Microscopic study of the brown ring syndrome. *J. Invertebr. Pathol.* **65,** 101-110.

Plana S., M. Le Pennec 1991. Altérations de la glande digestive et conséquences nutritionnelles chez la palourde *Ruditapes philippinarum* contaminée par une bactérie du genre *Vibrio. Aquat. Living Resour.* **4,** 255-264.

Riquelme C., G. Hayashida, A. E. Toranzo, J. Vilches, P. Chavez 1995. Pathogenicity studies on a *Vibrio anguillarum*-related (VAR) strain causing an epizootic in *Argopecten purpuratus* larvae cultured in Chile. *Dis. Aquat. Org.* **22,** 135-141.

Rodrick G. E., S. A. Ulrich 1984. Microscopical studies on the hemocytes of bivalves and their phagocytic interaction with selected bacteria. *Helgol. Meeresunters.* **37,** 167-176.

Schneeweiβ H., L. Renwrantz 1993. Analysis of the attraction of haemocytes from *Mytilus edulis* by molecules of bacterial origin. *Dev. Comp. Immunol.* **17,** 377-387.

Suresh K., A. Mohandas 1990. Number and types of hemocytes in *Sunetta scripta* and *Villorita cyprinoides var. cochinensis* (Bivalvia), and leukocytosis subsequent to bacterial challenge. *J. Invertebr. Pathol.* **55,** 312-318.

Wolmarans C. T., E. Yssel 1988. *Biomphalaria glabrata*: influence of selected abiotic factors on leukocytosis. *J. Invertebr. Pathol.* **51,** 10-14.

Yamamoto T., T. Yokata 1989. Adherence targets of *Vibrio parahaemolyticus* in human small intestines. *Infect. Immunol.* **57,** 2410-2419.