In vitro culture of mollusc hemocytes. Functional study of burst respiratory activity and analysis of interactions with protozoan and procaryotic pathogens

VIVIANE BOULO, DOMINIQUE HERVIO, ANNIE MORVAN, EVELYNE BACHERE and ERIC MIALHE

IFREMER, Unité de Recherche en Pathologie, Immunologie et Génétique Moléculaire, BP 133, 17390 La Tremblade, France

SUMMARY

The chief importance of hemocytes as immune effectors in molluscs has led to develop researches for *in vitro* culture.

The chemiluminescence technique has been used to estimate the phagocytosis capacity of *Crassostrea gigas* hemocytes according to different culture media and times. Sea water was used as a basic medium. Additives, such as antibiotics or glucose and buffers, such as Tris or Hepes, were tested as well as artificial sea water. Hemocyte chemiluminescence activities were recorded at t=0 in relation to the medium composition. The highest values were observed when hemocytes were kept in sea water without any modification. Whichever medium, chemiluminescence activity was substantially decreased when hemocytes were previously kept *in vitro* for a few hours. Thus, chemiluminescence technique could be a well-adapted method to estimate the functional capacity of mollusc hemocytes and to study medium composition in prospect of long-term molluscan cell cultures.

Despite present limitations of short-term hemocyte primary-cultures, they were used to study *in vitro* the interactions between hemocytes and some mollusc-specific pathogens.

The hemocyte recognition and entry mechanisms of *Bonamia ostreae* (Protozoa: Ascetospora), an intrahaemocytic parasite of flat oyster *Ostrea edulis*, were investigated. Moreover, it was demonstrated that hemocyte respiratory burst was not triggered during parasite phagocytosis.

Similar experiments were performed with St-Jacques scallop (*Pecten maximus*) hemocytes and a host-specific gill Rickettsiales-like organism, leading to evidence some pathogen adaptation for escaping host-immune response.

These first *in vitro* models will have to be progressively extrapolated to other pathogens in order to better understand anti-infectious immune response of molluscs.

INTRODUCTION

The first work in cell culture of marine molluscs date from 1960s (32). Many researches have been then performed to attempt medium composition improvements, specially by adding Vertebrates sera as a growth factor source (15, 30, 31). Progressively, the

preparation of primary cultures was getting more reliable, in particular because of aseptic treatments, tissue dissociation methods and medium composition frequently complemented with homologous and heterologous additives (11, 29). In spite of such improvements, neither marine mollusc cell line nor long-term primary cultures have yet been successfully established. This failure can be partially explained by the difficulty to eliminate some contaminatant microorganisms and protozoans and by the lack of data on mollusc cell physiology and biology. However, the chief difficulty for the evaluation of new medium compoments was related to available methods for cell viability analysis, essentially limited to light microscopy observations of primary cultures. Thus, *in vitro* cell survival appeared greatly variable according to its nature and degree of differenciation, relative good results being obtained with embryonic cells and hemocytes (18)..

The major importance of hemocytes as immune effectors in molluscs (5, 28) has led to apply primary cultures to develop *in vitro* researches on anti-infectious immunity. Phagocytosis has been widely investigated, essentially by light microscopy and generally using inert material or bacteria as stimulating agents (5). Recently, it has been shown that phagocytic processes are linked to oxidative metabolism (3, 12). Oxidative metabolic events, known as respiratory burst, are characterized by the production of active oxygen species (4, 9) These powerful oxidants are potentially effective as tumoricidal and anti-microbial agents and react with the internalized particles in various processes associated with oxidation-reduction reactions and generation of photons. This last phenomenon of light emission, can be detected by chemiluminescence.

The evidence of respiratory burst in oyster and scallop hemocytes (3, 12) has been realized according to chemiluminescence (CL) technique performed immediately after hemocyte withdrawal. This CL activity of hemocytes deserved interest as a possible technique for hemocyte function analysis in relation to culture medium composition and to primary cultures length. Moreover, the availability of purification protocols for some mollusc pathogens (16, 17, 14) permitted to develop *in vitro* researches on their interactions with hemocytes in terms of immunopathology.

RESULTS-DISCUSSION

Primary culture and assessment of functional capacity of Crassostrea gigas hemocytes.

Chemiluminescence was used as an analytical technique of hemocyte activity depending on phagocytosis of zymosan particles and related to lengths of previous *in vitro* culture (0 hr, 6 hrs, 18 hrs and 36 hrs). A protocol of chemiluminescence (CL) assays has been described (3), hemocytes being withdrawn using a modified Alsever solution (2) which avoids hemocyte aggregation as in the case of hemolymph or sea water. When maintained in this medium, cells stay in a quiescent state as estimated by the lack of

chemiluminescence activity after zymosan stimulation. By diluting the Alsever medium (2.5% final concentration) with sea water, the hemocytes recover their phagocytic activity as proved by light and electron microscopy or by CL technique (3).

The effect of culture medium on CL activity was investigated comparatively to sea water considered as a reference (Fig. 1); sea water with antibiotics: penicillin (150 µg/ml), bacitracin (150 µg/ml) and vancomycin (50 µg/ml); sea water with Tris-HCL (10 mM) or Hepes (20 mM); sea water with glucose (1mg/ml); artificial sea water (Sigma: Sea salts, 40 mg/ml). These media were selected because they permitted to tackle the effects of antibiotics and energetic molecules as well as pH on hemocyte activity and survival, the sea salts being considered because better-defined than sea water.

Immediately after the withdrawal and subsequent dilution with culture medium, hemocyte CL activity appeared twice in sea water compared to the other media (Fig. 1). After 6 hours of *in vitro* culture into these media, CL activity was yet observed for hemocyte primary cultures in sea water buffered with Hepes and lower values were recorded for sea water and sea water with glucose. Wnichever medium, no CL activity was detected after a 18-hour-length. Nevertheless, determination of viability by Trypan blue exclusion test showed that hemocytes stayed alive during several days in all the media. Moreover, whereas CL activity decreased strongly and quickly, light microscopy examinations showed that hemocytes were able to phagocyte zymosan particles, even after a 36-hour-length of *in vitro* culture without recording any CL response. Thus, hemocytes kept in these media lose very quickly their capacity of active oxygen species production. Improvements of culture medium are needed and will be possibly brought using CL as a suitable technique for qualitative and quantitative analyses of the effects of any product addition to medium composition. Other methods adapted to the analysis of cell functional capacity will have to be developed for mollusc hemocytes, specially microassays for metabolic enzymes.

Despite some limitations for long term experiments, hemocyte primary cultures in sea water may be already used as *in vitro* system for short-term studies about the interactions between immune cells and host-specific pathogens. Besides bacteria which are easily multiplied *in vitro*, intracellular microorganisms and parasites can be purified from infected molluscs (14, 16, 17, 23). Consequently, experiments about short-term hemocyte-pathogen interactions were interestingly undertaken with a special attention to recognition and phagocytosis mechanisms as well as the involvement of respiratory burst.

In vitro studies of hemocyte-pathogen interactions.

Rickettsiales-like organism (RLO) interactions with hemocytes of St-Jacques scallop, Pecten maximus.

Mass mortalities were reported in St-Jacques scallop, *Pecten maximus*, at Saint-Brieuc Bay (France). An intracellular procaryote

was observed inside parasitophorous vacuoles of gill endothelial cells (13). Ultrastructural characteristics and the lack of a complex developmental cycle related this procaryote with Rickettsiales. Histological investigations showed that the rickettsiales-like organisms (RLO) were frequently observed free in the hemolymph and that infected cells were generally clustered. These observations suggested limited spread of RLO with possible intervention of hemocytes and led us to consider interactions between the pathogens and the hemocytes. Purified RLO (14) and scallop hemocyte primary cultures permitted to undertake *in vitro* study of RLO-hemocyte interactions.

Electron microscopy for RLO phagocytosis assay. Hemocytes were withdrawn from the pericardic cavity and distributed into sterile Eppendorf tubes before adding purified RLO. After incubation (30 min; 1hr), the samples were processed for electron microscopy. The examination of these experimentally infected hemocyte primary cultures showed that purified RLO were quickly phagocytosed, since it appeared that numerous RLO were internalized as early as 30 minutes after contact. Some pictures suggested lysis of internalized RLO, particularly in samples fixed after one-hour incubation.

The RLO interactions with the respiratory burst of Pecten maximus hemocytes. Chemiluminescence (CL) activity of non-stimulated hemocytes was very low. After zymosan stimulation, the CL activity increased rapidly (Fig. 2A) and then decreased slowly over a few hours. These results indicated that *P. maximus* hemocytes produce oxygen radicals during phagocytosis. Moreover, Le Gall *et al.* (12) showed that the CL activity of *P. maximus* hemocytes was differentially inhibited by sodium azide (NaN3), which inhibits myeloperoxidase (21), and by catalase ,which has a quencher effect on singlet oxygen (21, 27).

When alive RLO were added to hemocyte primary cultures (Fig. 2B), no chemiluminescence response was observed. Similar results were obtained when RLO were previously killed by formalin (Fig. 2B).

Other experiments were performed with alive RLO (Fig. 2C), sonicated RLO (Fig. 2D) and killed RLO (Fig. 2E) added at the moment of chemiluminescence activity peak to hemocytes which were previously stimulated by zymosan. Whichever the kind of RLO treatment, this addition led to a decrease of hemocyte CL activity. Moreover, this decrease was more limited when RLO were also treated by L-tartrate which is an inhibitor of RLO acid phosphatase (24). These results suggest that RLO enzymatic may possess some enzymatic adaptations as yet proved for other intracellular pathogens (8, 19, 34).

Bonamia ostreae interactions with Ostrea edulis hemocytes

Bonamia ostreae (22) is an intracellular protozoan parasite of *Ostrea edulis* hemocytes, responsible of mass mortalities in oyster farming. Availability of infectious purified parasites made

interesting to develop an infection protocol of hemocyte primary cultures, taking into account parasitological and immunological perspectives since the dual role of hemocytes as host-cells for the parasite and as immune defense cells for the oysters.

Light and electron microscopy for B. ostreae phagocytosis assay. Hemocyte primary cultures were prepared from healthy flat oysters (*O. edulis*) collected in a bonamiasis-free area. Hemocytes were infected with purified *B. ostreae*, at a multiplicity of infection of 5, and fixed after different times of incubation. Electron microscopic pictures showed parasite engulfment by hemocyte pseudopods which progressively closed up in less than 30 min. Parasites appeared in almost all the hemocytic types (hyalinocytes or granulocytes), most generally into a parasitophorous vacuole suggesting an entry mechanism mediated by phagocytosis.

In order to confirm a phagocytic process, hemocytes were previously treated by cytochalasin B, a known inhibitor of microfilament function (1). This hemocyte treatment induced a strong reduction of infection rates whichever the hemocyte type (7), indicating an active role of hemocytes in phagocytosis. Host-directed phagocytosis (20), by some kind of "Zipper mechanism" (35) is likely to contribute to parasite entry. However, to unequivocally establish which is the reciprocal contribution of the hemocyte and of the parasite, complementary research is necessary, such as parasite inactivation by different treatments (33, 36).

Phagocytic entry raises questions relative to the existence of special ligands suceptible to bind *B. ostreae* to hemocytes and to induce recognition. Lectin-sugar interactions may be of importance in *B. ostreae* hemocyte interplay, as suggested by the presence of lectins on molluscan hemocyte membrane (25).

The availability of anti-*B. ostreae* monoclonal antibodies (26), combining with parasite membrane epitopes (6), has allowed experiments showing partial inhibition of entry into hemocytes (data not shown) suggesting a role for parasitic receptors.

Another line of questions concerns the development of the parasite once inside hemocytes, whose main functions are to destroy phagocytosed bodies. Such experiments would be based on long-term hemocyte primary cultures which will depend on improvements of *in vitro* culture of mollusc cells.

B. ostreae interactions with respiratory burst of O. edulis hemocytes. CL activity of hemocytes was studied after stimulation with *B. ostreae* purified cells. The results showed in Fig. 3 indicate that no activity was recorded when *B. ostreae*, either alive or killed, enter into hemocytes, while hemocytes stimulated by zymosan particles have a high CL activity. This lack of respiratory burst activation could correspond to an adaptation of the parasite to intracellular survival and development. This emphasizes the need for research based upon *B. ostreae* enzymatic equipment, with a special attention to enzymes possibly involved as inhibitors of respiratory burst, such as acid phosphatase already inside characterized *B. ostreae* cells (10).

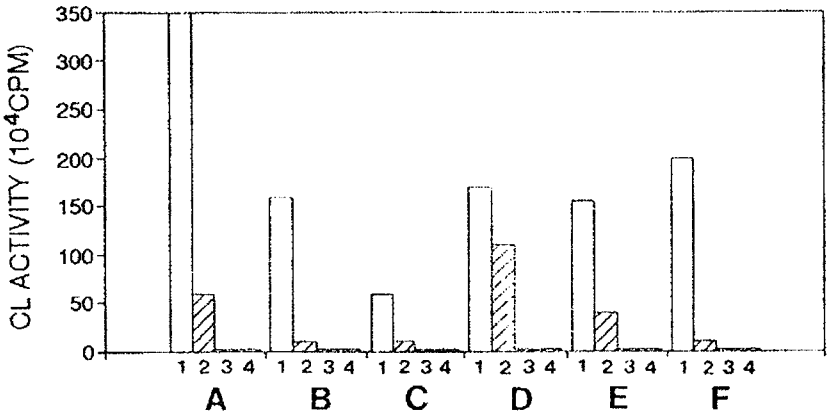

Fig. 1: Chemiluminescence activity of *Crassostrea gigas* hemocyte primary cultures stimulated by zymosan according to culture lengh (1=0 hr; 2=6 hrs; 3=18 hrs; 4=36 hrs) and media, A: sea water; B: sea water with antibiotics; C: sea water with Tris-HCL; D: sea water with Hepes; E: sea water with glucose; F: sea salts (sigma).

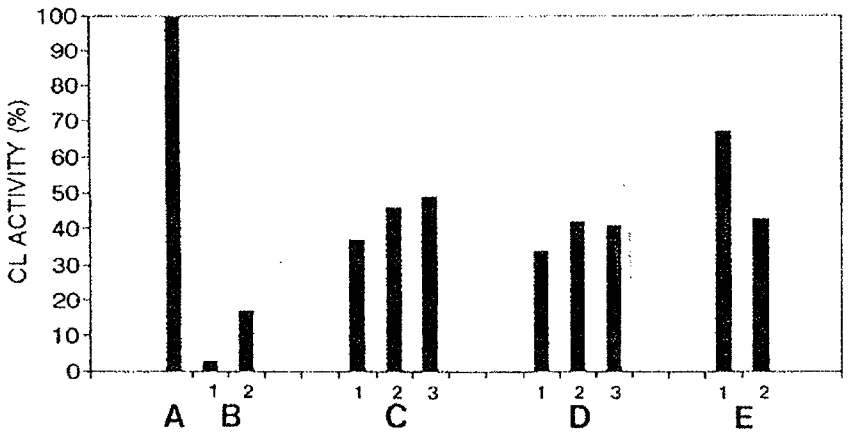

Fig. 2: CL activities of *Pecten maximus* hemocytes stimulated with zymosan (A) or with RLO (B), (alive (1) or killed (2)). Relative CL activites of *Pecten maximus* hemocytes stimulated with zymosan previously to the addition of alive RLO (C) (untreated (1); L-tartrate (2) or NaN3 (3)); sonicated RLO (D) (untreated (1); L-tartrate (2) or NaN3 (3)) and killed RLO (E) (Formalin (1) or heat (2)).

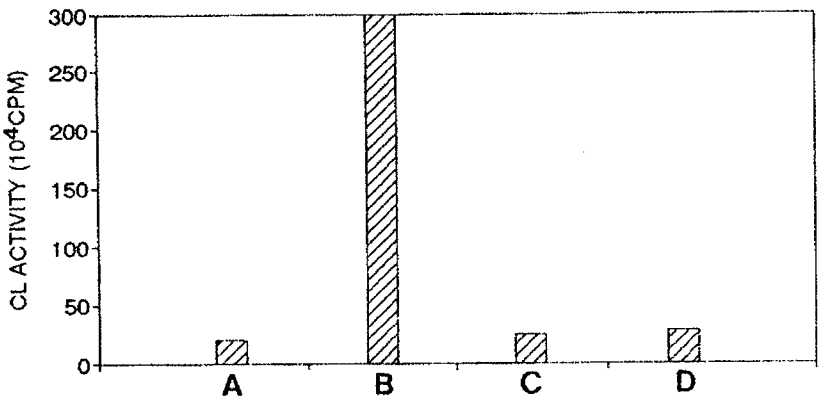

Fig. 3: Chemiluminescence activity of *Ostrea edulis* hemocytes stimulated by zymosan (B) and *Bonamia ostreae*, alive (C) or heat-treated (D); A: non-stimulated control hemocytes.

CONCLUSION

These experiments show that hemocytes of *P. maximus* and *O. edulis* produce oxygen radicals during phagocytosis, as do vertebrate phagocytic cells. Similar results have been obtained for *Mytilus edulis* hemocytes (Noël, pers. comm.) but no CL activity has been recorded for *Ruditapes philippinarum* hemocytes (Lopez, pers. comm.). The *in vitro* studies of oxygen-dependent killing mechanisms by CL technique show that pathogens, such as Rickettsiales-like procaryotes and Ascetospora protozoans, have some efficient adaptations for avoiding or counteracting host microbicidal response. However, the conditions of *in vitro* culture of hemocytes will have to be improved, in terms of viability and functionality, because long-term primary cultures are necessary to better analyse the fate of internalised pathogens. In this prospect, CL will be advantageously used for objective analysis of culture medium composition.

The availability of hemocyte reliable primary cultures would also permit to study activation of microbicidal activity by heterologous molecules, such as cytokines (TNF, interleukin), in a more fundamental and comparative approach of immunology.

From now, hemocyte primary cultures can be used in short-term experiments for studying the parasite/hemocyte interactions of other mollusc species and other pathogens.

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