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Chemiluminescence analysis of the activity of *Pecten maximus* hemocytes stimulated with zymosan and host-specific Rickettsiales-like organisms

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ABSTRACT: During a study of Rickettsiales-like organisms (RLO) in *Pecten maximus* gill, histological observation of free RLO in hemolymph led us to consider interactions between the pathogen and the hemocytes. Firstly, the production of oxygen radicals by hemocytes was established using a luminol-enhanced chemiluminescence method with zymosan as stimulant. The effects of several inhibitors of oxidative metabolism were investigated. The internalization of RLO into hemocytes was demonstrated by electron microscopy but was not related to chemiluminescent activity. The possible involvement of RLO acid phosphatase as the inhibitor of respiratory burst was assessed.

INTRODUCTION

At the end of each winter from 1986 to 1988, mass mortalities were reported in *Pecten maximus* beds at Saint-Brieuc Bay (North Brittany). A histological study, performed in March 1987, on different tissues of bay scallops from Saint-Brieuc Bay found Rickettsiales-like organisms (RLO) in parasitophorous vacuoles of gill endothelial cells (Le Gall et al. 1988). Because several cases of RLO have been associated with mortalities of bivalve species (Buchanan 1978, Comps & Raimbault 1978) and particularly of scallops (Gulka et al. 1983), the *Pecten maximus* RLO were considered to be involved in the recent mortalities.

Moreover, these histological investigations showed that RLO were frequently observed free in the hemolymph, and that infected cells were generally clustered. These observations have to be considered in regard to host-pathogen interactions. Indeed, the free RLO, discharged from disrupted infected cells, may be involved in cell-to-cell transmission of microorganisms. Their momentary localization in hemolymph raised the question of their interactions with host hemocytes. In Mollusca, hemocytes represent the primary effector component in defense functions, by using phagocytosis or encapsulation (Bang 1975, Anderson 1977, Sminia & Van Der Knaap 1987). Recently, the production of toxic oxygen radicals has been histochemically demonstrated for hemocytes of *Patinopecten yessoensis* (Nakamura et al. 1985); it can also be detected by chemiluminescence in response to a phagocytic stimulus. This phenomenon, originally described by Allen et al. (1972), is the result of the production of toxic free oxygen radicals by oxidative metabolism and is known as respiratory burst. Oxidative metabolism constitutes 1 of the 2 antimicrobial mechanisms established in the vertebrate phagocytic process, the other one being related to the enzymatic process (Thomas et al. 1988).

The generation of oxygen radicals is currently investigated by luminol-enhanced chemiluminescence (CL), a method which amplifies the native light emitted by excited molecules (Allen & Loose 1976, Prendergast & Proctor 1981). By referring to vertebrate phagocytic cells (Roos 1980), this methodology has been developed for several gastropod species – *Lymnaea stagnalis* (Dikkeboom et al. 1987), *Planorborius corneus, Helix aspersa* (Dikkeboom et al. 1988) and *Biomphalaria glabrata* (Shozawa 1986) – and recently for oysters (Larson et al. 1989, Bachère et al. 1991).

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The present work aims at analysing the respiratory burst of *Pecten maximus* hemocytes by CL and focuses on interactions of the hemocytes with host-specific RLO, taking into account the frequent observation of free procaryotes in hemolymph in the course of infections.

MATERIALS AND METHODS

Specimens. For purification of RLO and hemolymph collection, 2- to 3-yr-old bay scallops were dredged from the bay of Saint-Brieuc (Brittany), an area which has been chronically affected by rickettsiosis for 4 yr.

Parasites. RLO were isolated and purified from infected bay scallop gills by successive differential and isopycnic centrifugations. Purified parasite viability was verified using an acridine orange and ethidium bromide assay (Parks et al. 1979). Parasite numbers were determined microscopically using a Mallassez blood-counting cell.

Hemolymph collection. The scallops were kept opened by force and their bodies were thoroughly washed with sterile seawater (SSW). Hemolymph was then withdrawn from the pericardial cavity and simultaneously diluted 1/1 in modified anti-aggregant Alsever solution (Bachère et al. 1988).

Chemiluminescence assay. ***General protocol:*** Generation of chemiluminescence was measured with a liquid scintillation counter (Tri-Carb 2200 CA Packard) in the out-of coincidence mode. The counter was set on repeated cycles and sequential counts were made at 0.5 min intervals during 5 h periods. All the assays were performed at room temperature in plastic scintillation vials containing the hemocytes suspended in a final volume of 2 ml completed with SSW. The luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), stored (0.1 M) in dimethyl sulfoxide (DMSO), was diluted in SSW immediately before use to obtain a final concentration of 10^{-4} M. It has been shown that this low concentration of DMSO has no effect on the CL response (Bachère et al. unpubl. results). The CL base activity of the hemocytes was recorded for a run before adding the stimulants. A non-stimulated control prepared from the same hemolymph sample was always analysed in parallel.

Experimental parameters: For stimulation of hemocyte phagocytic activity, zymosan particles (Zymosan A, Sigma) at 40 mg 10 ml^{-1} were suspended in SSW and boiled for 30 min, washed twice and then resuspended in SSW before being divided into aliquots and stored at −20°C. The aliquots were thawed and counted in a Mallassez cell immediately before use. The CL experiments were performed with 80 zymosan particles per hemocyte, this ratio eliciting an optimal response (Le Gall et al. 1989).

Hemocyte quantity: For all the experiments, a set of vials was prepared, each containing 2×10^5 hemocytes from the same hemolymph sample collected into an equal volume of Alsever solution. Because Alsever solution concentration interferes with the CL response unless the final concentration is under 2.5% (data not shown), only those hemolymph withdrawals containing more than 2×10^6 hemocytes ml^{-1} (hemolymph/Alsever) were used. These samples were diluted with SSW to give 2×10^5 hemocytes in 2 ml, this final volume comprising luminol and the stimulant suspension (zymosan or RLO).

Inhibition of phagocytosis: The inhibitory effect of cytochalasin B was determined at a final concentration of 10 µg ml^{-1} by addition to the hemocyte suspension just before starting the phagocytic stimulation.

Luminol-enhanced CL inhibitors: Effects of some luminol-enhanced CL inhibitors were determined by adding each to the assay vials at the time of the CL peak induced by zymosan stimulation. The final concentrations of inhibitors tested were 80 and 10 µg superoxide dismutase (SOD) ml^{-1}, 65 µg sodium azide (NaN_3) ml^{-1} (10^{-3} M) and 65 µg potassium cyanide (KCN) ml^{-1} (10^{-3} M). Hemocyte viability after incubation in these different solutions was determined with an acridine orange/ethidium bromide test.

Stimulation with RLO: Hemocytes, after being stimulated with zymosan particles or left unstimulated, were stimulated with purified *Pecten maximus* RLO at a ratio of 80:1 RLO:hemocyte (Le Gall et al. 1989). Some assays were performed with live RLO, preincubated for 30 min in sodium L-tartrate (3.3×10^{-6} mol ml^{-1}) or in NaN_3 (3×10^{-4} mol ml^{-1}). Other assays were similarly performed with RLO killed by formalin (1%) or by heat (100°C for 20 min), or with homogenates of RLO disrupted by ultrasound (100 W, 60 s).

Electron microscopy for RLO phagocytosis assay. Hemolymph samples (300 µl) were distributed in sterile Eppendorf tubes into which RLO were added (particle:hemocyte ratio of 50:1). After incubation (30 or 60 min, 18°C), the samples were fixed with an equal volume of 1.25% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate-HCl buffer (pH 7.4), whose osmolarity was raised to 1000 mOsmol with sucrose. Cells were rinsed 3 times with iso-osmotic phosphate-buffered saline and post-fixed in 1% osmium tetraoxide (30 min). After another wash, the pellets were preembedded in 1% agarose (Low Gel Temperature, Bio Rad) and processed for Epon 812 embedding by using an LKB Automatic Processor. Ultrathin sections (60 to 90 nm) were double-contrasted with aqueous uranyl acetate and lead citrate (LKB Ultrostainer) and observed under a Jeol 1200 CX electron microscope.

RESULTS

Hemocyte chemiluminescence activity

Luminol-enhanced CL methodology was applied to search for *Pecten maximus* hemocyte oxidative metabolism using zymosan particles as phagocytosis stimulants. Whatever the assay, control and experimental samples were strictly equivalent since they were prepared from the same withdrawn hemolymph sample.

Results obtained with hemocytes from 3 scallops are shown in Fig. 1. Base activities were very low for non-stimulated hemocytes. After zymosan stimulation, CL activities increased rapidly, reaching maximum values after about 1 h, and then decreased slowly over several hours. Although the CL response patterns were similar, some intensity and duration differences were observed in individual scallops.

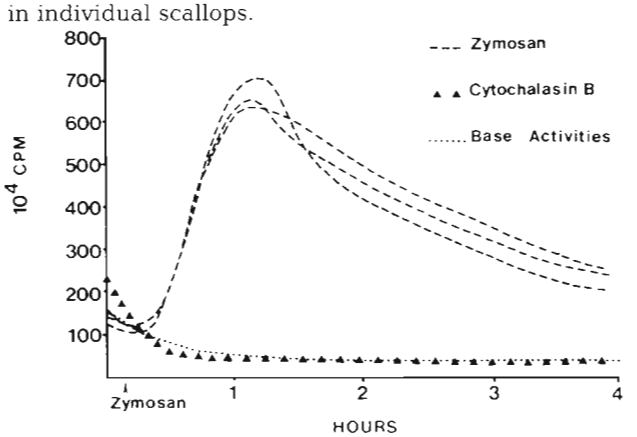

Fig. 1. *Pecten maximus*. Chemiluminescent responses of scallop hemocytes stimulated by zymosan (---) (particle : hemocyte ratio 80 : 1; 3 different scallops); pretreated by Cytochalasin B (10 µg ml^{-1} final conc.) and then zymosan-stimulated (▲▲▲▲); non-stimulated (·····)

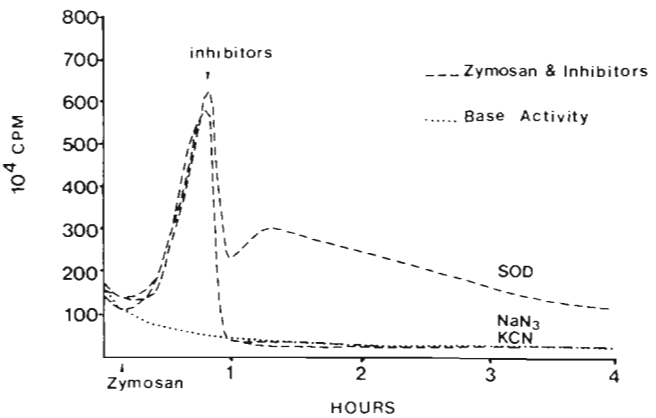

Fig. 2. *Pecten maximus*. Effect of inhibitors on chemiluminescent responses of scallop hemocytes stimulated by zymosan (particle : hemocyte ratio 80 : 1); inhibitors were 80 µg SOD ml^{-1}, 10^{-3} M NaN_3, and 10^{-3} M KCN. (·····) Non-stimulated

The effect of cytochalasin B on hemocyte CL activity was then examined because of the capacity of this reagent to prevent phagocytosis (Stuart et al. 1985). Preincubation of hemocytes with cytochalasin B (10 µg ml^{-1}) led to a total suppression of CL response, showing the close connection between phagocytosis and hemocyte CL activity (Fig. 1).

Several respiratory burst inhibitors were tested, each hemocyte sample set being prepared from a single scallop. The addition of exogenous SOD to stimulated hemocytes caused no effect at a concentration of 10 µg ml^{-1}. However, 80 µg SOD ml^{-1} decreased CL activity to 50 % (Fig. 2). Hemocyte viability was confirmed for this last SOD concentration. With sodium azide (NaN_3) at 10^{-3} M, the hemocytes remained viable but their CL activity was immediately and completely suppressed (Fig. 2). KCN also suppressed CL response but, at the tested concentration (10^{-3} M), hemocyte viability was reduced by half.

Hemocyte-RLO interactions

Before studying RLO interaction with the respiratory burst of *Pecten maximus* hemocytes, we had examined cells by electron microscopy to determine if purified microorganisms were indeed phagocytosed. For this purpose, hemocytes were infected in vitro with purified parasites, the cells being fixed after 30 min and 1 h incubation. It appeared that RLO were phagocytosed as early as 30 min after introduction (Fig. 3), some hemocytes containing several microorganisms, sometimes included in the same parasitophorous vacuole. Some micrographs suggested lysis of RLO, particularly in samples fixed after 1 h incubation.

When live pathogens were added to vials containing hemocytes which had not been previously stimulated, no CL response was detected. Addition of live pathogens at maximal CL activity of hemocytes previously stimulated with zymosan led to a 45 to 74 % decrease in luminescence (Table 1). A pretreatment of live RLO with sodium L-tartrate (3.3×10^{-6} mol ml^{-1}) or with NaN_3 (3×10^{-4} mol ml^{-1}) led to smaller decreases in hemocyte CL activity, ranging between 50–53 % and 41–56 % respectively (Table 1).

When RLO killed by formalin were added to hemocytes, no CL activity was noticed. However, if they were added to zymosan-stimulated hemocytes at their CL activity peaks, CL activity was quickly reduced (by 28.6 to 31.5 %) to a lower level than with live RLO (Table 1). If the parasites had been killed by heat, a more marked decrease was observed (51 to 58.5 %; Table 1).

When RLO homogenate was added to zymosan-stimulated hemocytes, CL activity was decreased by

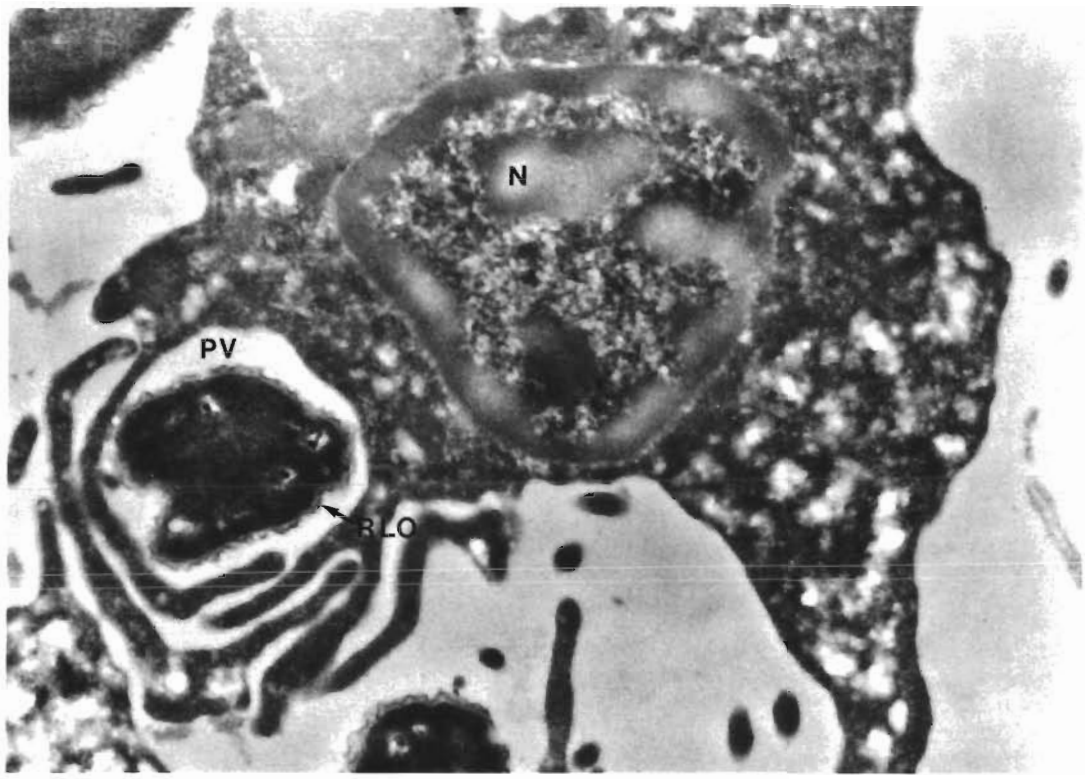

Fig. 3. *Pecten maximus*. Transmission electron micrograph of scallop hemocytes infected with RLO (Rickettsiales-like organism) cells and fixed 30 min after parasite challenge, showing progressive engulfment of RLO into a phagocytic vacuole (PV); N: hemocyte nucleus. (× 16 000)

Table 1. *Pecten maximus*. Effect of different preparations of Rickettsiales-like organisms (RLO) on CL responses of scallop hemocytes previously stimulated by zymosan (80:1), expressed as percentage of inhibition. Live or homogenated RLO were untreated (Untr.) or pretreated with 3.3×10^{-6} mol L-tartrate ml^{-1} or 3.3×10^{-4} mol NaN_3 ml^{-1}. RLO killed by formalin or by heat

Expt	Live			Killed		Homogenated		
	Untr.	L-tart.	NaN_3	Formalin	Heat	Untr.	L-tart.	NaN_3
1	72.0	–	–	28.6	–	–	–	–
2	74.0	–	–	31.5	–	–	–	–
3	48.0	50.0	41.0	–	58.5	–	–	–
4	45.0	53.0	56.0	29.0	51.0	63.0	55.0	56.0

63 %; when the homogenate had been preincubated in sodium L-tartrate (3.3×10^{-6} mol ml^{-1}) or in NaN_3 (3×10^{-4} mol ml^{-1}), activity decreased by 55 and 56 % respectively (Table 1).

DISCUSSION

This in vitro study of interactions between RLO and host was initially based on an analysis of oxidative metabolism in *Pecten maximus* hemocytes. The experimental parameters established for oyster hemocytes, using hemolymph withdrawals with antiaggregant solution and low (10^{-4} M) luminol concentration (Bachère et al. 1991), were perfectly suited for scallop hemocytes. The number of hemocytes contained in each counting vial was fixed at 2×10^5 because good CL signals were obtained and several similar samples from a single hemolymph withdrawal could be prepared. Consequently, by comparing CL activities of hemolymph samples from a single scallop, inter-individual variability was excluded. Thus, if the observed variation in individual CL activities was not caused by methodological factors, it may be suggested that qualitative differences in hemograms were the cause. This hypothesis could be tested by analysing CL activity of each hemocyte type, separated beforehand by isopycnic centrifugation and centrifugal elutriation (Bachère et al. 1988), or by immunopurification using specific monoclonal antibodies.

The CL activity of *Pecten maximus* hemocytes was differentially inhibited by SOD, NaN_3 and KCN. SOD is known to catalyse the decomposition of superoxide anion (O_2^-) into O_2 and H_2O_2 (McCord & Fridovich 1969). The CL inhibition observed when SOD was used at 80 µg ml^{-1} (50 %) (as opposed to no effect at 10 µg ml^{-1}) is consistent with data concerning luminol-enhanced CL for human neutrophils (De Chatelet et al. 1982), murine polymorphonuclear granulocytes and macrophages (Müller-Peddinghaus 1984, Vincendeau et al. 1988) and gastropod hemocytes (Dikkeboom et al. 1987, 1988). NaN_3 inhibits myeloperoxidase (MPO) (Nakagawara et al. 1981) and catalase, and has a quencher effect on singlet oxygen ($^1O_2$) (Rosen & Klebanoff 1977). At similar tested concentrations, the results obtained for *P. maximus* hemocytes were in agreement with those previously cited. Also, the complete inhibition of CL by KCN, an inhibitor of SOD, MPO and catalase (McCord & Fridovich 1969, Nakagawara et al. 1981), corresponds to data acquired by De Chatelet et al. (1982) and Müller-Peddinghaus (1984) in human and murine phagocytes. Therefore, these results indicate that *P. maximus* hemocytes produce, during phagocytosis, oxygen radicals as do vertebrate phagocytic cells.

Because the reactive oxygen species are well known in vertebrates as strong microbicidal parasiticidal effectors (Klebanoff 1968, 1982, Babior 1978, Nakagawara et al. 1981), their involvement in rickettsiosis specific to *Pecten maximus* was worth analysing. Indeed, although RLO infect the gill endothelial cells, they have an extracellular phase in the hemolymph which may be related to the infection process of new cells. Moreover, infections are quickly established and heavy in young scallops reared in infected areas, which suggests that their defense mechanism is relatively ineffective. Prior to CL experiments, the phagocytosis of rickettsias by *P. maximus* hemocytes was established by electron microscopy. Some micrographs suggested an intravacuolar disintegration of the RLO.

Despite this patent in vitro phagocytosis process, chemiluminescence experiments showed no production of free oxygen radicals by RLO-stimulated hemocytes. Thus, either RLO do not trigger cell respiratory burst or they possess a highly efficient scavenger system. This last hypothesis was supported by experiments with zymosan-stimulated hemocytes, subsequently incubated with live, killed or homogenated parasites. A substantial interference of the host hemocyte oxidative metabolism was observed, whatever the type of RLO suspension. The relatively slight inhibition shown by heat- or formalin-killed parasites compared to live or homogenized parasites suggested denaturation of the CL inhibitors.

Many intracellular pathogens associated with macrophages are adapted enzymatically to survive in this deleterious environment. Enzymes such as SOD, catalase (Weiss et al. 1987) and glutathione peroxidase (Mkoji et al. 1988) have been shown to play a prominent role in protecting parasites against host respiratory burst and may be responsible for this adaptive strategy. It is also suggested that acid phosphatase, recently demonstrated in *Pecten maximus* RLO (Le Gall et al. unpubl. data) and particularly well-studied in *Leishmania* parasites (Gottlieb & Dwyer 1981, Glew et al. 1982), reduces the ability of the phagocytic cell to produce superoxide anions (Remaley et al. 1984). In CL experiments, inhibition of *P. maximus* hemocyte respiratory burst was decreased if RLO were incubated with L-tartrate, an inhibitor of some acid phosphatases. The influence of NaN_3 on hemocyte respiratory burst inhibition may be the result of a pathogen peroxidase. However, its function will have to be defined by chemical characterization.

In conclusion, the interactions between scallops and RLO can now be analysed from an immunological point of view using experiments at the cellular level. It is possible to estimate the defense mechanism of individual scallops without killing them by using CL techniques performed on hemolymph samples. Such a methodology is particularly suitable for investigating the hemocyte responses of scallops of different ages. Indeed, transmission of rickettsial-like infection is horizontal, and juveniles become infected when placed in a contaminated area (Le Gall et al. 1991). It is possible that juvenile sensitivity is correlated with an undifferentiated defense mechanism system, as suggested by Leibovitz (1989) for chlamydiosis in *Argopecten irradians*. This disease is fatal for juveniles, whereas only mild for adults.

The mechanism of inhibition of host hemocyte respiratory burst by RLO needs to be defined in more detail. The actual survival of parasites should be analysed using long term in vitro culture of hemocytes infected with purified procaryotes. However, the preliminary results described in this paper may explain the short-term survival in hemolymph, which is required by rickettsias for dispersal to host cells after disruption of parasitophorous vacuoles.

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