Luminol-dependent chemiluminescence by hemocytes of two marine bivalves, *Ostrea edulis* and *Crassostrea gigas*

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ABSTRACT: Microbicidal phagocytic function and oxidative metabolism of the hemocytes of 2 oyster species, *Ostrea edulis* and *Crassostrea gigas*, were investigated using a luminol-enhanced chemiluminescence (CL) technique. First, experimental parameters adapted to marine bivalve hemocytes were established on hemolymph pools of *C. gigas* in order to obtain qualitatively and quantitatively homogeneous samples and so to perform statistically comparable assays. The use of Modified Alsever Solution (MAS) allowed hemocytes to be kept non-aggregated and in a non-stimulated state until starting the assays. A number of aliquots of 2×10^5 hemocytes with an MAS final concentration of 3.5 % showed high chemiluminescent response after stimulation by zymosan particles. A particle:hemocyte ratio of 80:1 gave optimal CL activity. Activity was inhibited by cytochalasin B, a phagocytic inhibitor, and by sodium azide, the latter indicating the involvement of oxygen products. Having thus defined suitable parameter values, the CL protocol was then applied to *O. edulis* for qualitative and quantitative analyses of respiratory burst capacity at the species and individual levels. Results suggested inter- and intra-specific variability of CL responses. Under the same experimental conditions, *O. edulis* hemocytes generally displayed higher CL activities than *C. gigas*. Moreover, clear individual variability was demonstrated. The same experimental number of hemocytes showed great differences in CL responses between individuals suggesting a possible correlation with hemogram characteristics such as cell type percentages.

INTRODUCTION

The phagocytic functions of mollusc hemocytes have been widely investigated by optical and electron microscopy, generally using inert material or bacteria as stimulating agents (Bang 1975), and more recently native pathogens because of the availability of specific isolation and purification protocols (Mialhe et al. 1988). These observations led us to compare mollusc hemocytes with immune cells of better-studied animal species in which phagocytosis constitutes an important way of control and elimination of a wide range of pathogens, microorganisms and tumor cells. In vertebrates, phagocytic processes are linked to subsequent microbicidal activities which rely on an oxygen-independent system (Spitznagel 1984) and on oxidative metabolism (Andrew et al. 1985, Thomas et al. 1988). Oxidative metabolic events, known as respiratory burst, are characterized by the production of active oxygen species (Badwey & Karnosky 1980, Halliwell & Guteridge 1984). These powerful oxidants are effective as tumoricidal and antimicrobial agents and react with the internalized particles in various processes associated with oxidation-reduction reactions and the generation of photons. This last phenomenon of light emission, which can be detected in chemiluminescence (CL), is frequently used for studying phagocytosis and cell-mediated cytotoxicity in known phagocytes such as neutrophils, eosinophils and mononuclear cells (Allen et al. 1972, Trush et al. 1978).

CL may be regarded as having acquired the status of reference methodology in immunology, since it provides an in vitro measure of the immune state of an animal (Fromtling & Abruzzo 1985). The existence of this methodology led to the application of CL to invertebrates, notably to several medically important gastropod species, in order to assess their immune response against transmitted parasites such as *Schistosoma* (Dikkeboom et al. 1988a). Thus, the ubiquitous feature of respiratory burst was discovered in molluscs. In the scallop *Patinopecten yessoensis* (Bivalvia) respiratory burst phenomena were demonstrated by a histochemical method (Nakamura et al. 1985).

This paper describes the establishment of experi-

mental parameters suitable for luminol-enhanced CL assays on hemocytes of the marine bivalve *Crassostrea gigas*. CL was applied to qualitative and quantitative analyses of the respiratory burst in order to validate the hemocyte phagocytic response as a potential monitor of the oyster defense capacity. The standardization of the method permitted study of individual CL responses in 2 bivalve species, *C. gigas* and *Ostrea edulis*. Moreover, following the example of vertebrates, for which blood cell types exhibit different functions, a correlation was sought between CL activities and hemogram characteristics as an aid to understanding the variability of oyster individual response.

MATERIALS AND METHODS

Animals. Three-year-old flat oysters *Ostrea edulis* were collected from the Etang de Thau, France, (Mediterranean Sea) which is an area free of parasites *Bonamia ostreae*. Three-year-old Japanese oysters *Crassostrea gigas* originated from Marennes-Oleron, France (Atlantic Ocean).

Hemolymph sampling. Hemolymph samples were collected from the pericardial cavity and, generally, simultaneously 2-fold diluted with anti-aggregant Modified Alsever Solution (MAS) (Bachère et al. 1988). The hemolymph samples were used either separately or pooled (10 individual hemolymphs).

Hemogram determination. Hemocyte counts were performed, using a Malassez hemacytometer, on hemolymph samples diluted in MAS as just described. For the estimation of numbers of different hemocyte types, a second blood withdrawal from the same individual without MAS was necessary to avoid disruption of slide-adherent hemocytes by the MAS. The pure hemolymph samples (50 µl) were deposited on microscope slides and 50 µl of filtered seawater (FSW) was added. The hemocytes were allowed to settle for 30 min at room temperature in a moist chamber. Then, the hemocytes were identified by vital staining using the acridine orange ethidium bromide method (Herzenberg 1979). Briefly, 100 µl of the test mixture in FSW was added to the settled hemocytes, which were then observed with an epifluorescence microscope. After counting 200 hemocytes, the percentages of granulocytes and small and large hyalinocytes were estimated.

Zymosan. Zymosan particles (Sigma, USA) were used as a phagocytic stimulus. They were suspended (40 mg ml^{-1}) in FSW, heated for 30 min at 100°C, washed and finally suspended in FSW, aliquoted and stored at -20°C. At the time of use, the exact number of zymosan particles, in a thawed aliquot, was estimated using a Malassez hemacytometer

Phagocytosis assays. Phagocytosis assays were performed by adding 100 µl of a zymosan suspension to adherent hemocytes on microscope slides, prepared from pure hemolymph samples. After about 30 min incubation, the preparations were washed with FSW, fixed with methanol and finally stained with Hemacolor Kit (Merck). Similar assays were made with hemocyte preparations from MAS-diluted hemolymph samples, washed in advance and resuspended in FSW. In each preparation, the phagocytic activity of the hemocytes was studied by light microscopy.

Chemiluminescence assays. *General protocol:* Generation of CL 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 0.5 min counting periods at fixed intervals, generally for 5 h. The assays were performed at room temperature. Each plastic scintillation vial contained 2×10^5 hemocytes obtained by diluting an adequate quantity of a hemolymph/MAS (1:1) sample with FSW. Luminol (3-aminophtalhydrazide, Sigma, USA) solutions were prepared just before use from a stock solution [10^{-1} M in DMSO (dimethyl sulfoxide) Sigma] and 200 µl was introduced into each vial, in a total volume of 2 ml. The CL base activity of the hemocytes was recorded for a first run. Then, the zymosan suspension was added at a selected zymosan particle-to-hemocyte ratio and the CL responses were measured. Generally, a vial was kept without stimulus as a control for the measurement of CL base activity during the experiments.

Experimental parameters: The experimental parameters were established with *Crassostrea gigas* hemocytes.

Effect of MAS concentration. Hemolymph samples were obtained as previously described but diluted 2-fold in FSW instead of MAS. The CL assay was then performed, according to the general protocol, with 2×10^5 hemocytes, stimulated by zymosan (with a particle:hemocyte ratio of 80:1) and with luminol at a final concentration of 10^{-4} M. CL measurements were recorded for these stimulated hemocytes in parallel with non-stimulated ones.

The hemolymph sample withdrawn in MAS from one oyster (2×10^6 hemocytes ml^{-1}) was distributed at 100 µl (2×10^5 hemocytes) per vial. Then, appropriate quantities of MAS and FSW were added in order to obtain, in a 2 ml final volume, an MAS concentration series corresponding to 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5 and 10.5 %. For the assay, the final concentration of luminol was 10^{-4} M, and the hemocytes were stimulated by zymosan at a ratio of 80:1.

Effect of luminol concentration. From a 10^{-1} M luminol stock solution, a concentration series was prepared in FSW in order to obtain luminol concentrations of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 0 M luminol. Hemocyte/

MAS samples (2×10^5 hemocytes vial^{-1}, 2.5 % MAS), all obtained from pooled hemolymph from 10 oysters, were stimulated by zymosan at a ratio of 80:1.

Effect of zymosan stimulus dose. The CL responses of the hemocytes of pooled hemolymph from 10 individuals were measured after stimulation at different zymosan particle:hemocyte ratios, viz. 20:1, 40:1, 60:1, 80:1, 100:1, 120:1, 140:1, 160:1. The luminol concentration was 10^{-4} M.

Inhibition assays with Crassostrea gigas hemocytes: The inhibitory effect of cytochalasin B (Sigma; 1.5 µg ml^{-1}) was determined by adding 3 µl of stock solution (1 mg ml^{-1} in DMSO) to the hemocyte suspensions just before starting the phagocytic stimulation. The effect of sodium azide (NaN$_3$, Sigma; 32 µg ml^{-1}) was determined by adding 5 µl of stock solution when the CL responses reached the peak activity. These experiments were performed according to the general protocol: 2×10^5 hemocytes vial^{-1} obtained from pooled samples of hemolymph/MAS; MAS 3 % final concentration; luminol 10^{-4} M; zymosan particle:hemocyte ratio of 80:1.

CL assays of individual hemolymph samples: Hemolymph samples were individually withdrawn, simultaneously diluted 2-fold in MAS, from 10 oysters of each of the 2 species. The CL assays were performed according to the general protocol established with *Crassostrea gigas* hemocytes previously described.

RESULTS

Phagocytosis assay

Microscopical observations of glass-adherent hemocytes from undiluted samples showed a quick internalization of zymosan particles which were observed inside the hemocytes as soon as 5 min after addition. The 2 main types of cells, hyalinocytes and granulocytes of both oyster species, were able to phagocytose yeast walls.

When hemocyte preparations were made from hemolymph collected with MAS (1:1) and subsequently washed in FSW, the phagocytic capacity was similar to that of hemocytes from pure hemolymph.

Chemiluminescence experimental parameter selection with *Crassostrea gigas* hemocytes

Effect of MAS concentration on CL. The withdrawal of hemolymph samples, without anti-aggregant MAS, directly into FSW led to immediate high values (2.7×10^6 cpm) of CL activity even without zymosan stimulation. When zymosan stimulation was subsequently applied, a lower peak value (1.8×10^6 cpm) was obtained, i.e. there was a rapid decrease in the signal. For hemolymph samples collected with MAS (1:1), the CL basis activities of the hemocytes were always very low, about 0.25×10^6

cpm. The CL responses of such hemocytes after stimulation by zymosan varied in intensity according to the MAS concentration (Fig. 1): whereas the CL peak intensities were respectively 1.92×10^6 cpm and 1.84×10^6 cpm for 2.5 % and 3.5 % MAS, they were strongly reduced between 4.5 and 10.5 %, decreasing from 0.7×10^6 to 0.4×10^6 cpm. Whatever the MAS concentration, low CL values were observed for the corresponding unstimulated samples (about 0.25×10^6 cpm).

Effect of luminol concentration on CL. Comparison of the different luminol-enhanced assays showed a maximal response for 10^{-4} M luminol (3.6×10^6 cpm with the hemolymph pool assayed) (Fig. 2). At the next higher concentration tested (10^{-3} M), the CL peak intensity attained only 0.7×10^6 cpm. At the next lower concentration (10^{-5} M), low CL responses were also observed, 0.8×10^6 cpm. No CL activity was obtained without luminol, nor at the concentrations of 10^{-2} M and 10^{-6} M. Therefore, subsequent experiments were all performed at 10^{-4} M final luminol concentration, the equivalent 10^{-3} M DMSO concentration being without effect on chemiluminescence (data not shown).

Effect of zymosan particle:hemocyte ratio on CL. As previously shown, the contact of hemocytes with zymosan particles led to CL responses. These responses reached a peak generally about 60 min after contact and then decreased slowly over 4 h. However, the level and swiftness of CL response were dependent on the number of zymosan particles added (Fig. 3). A maximum CL activity of 3.84×10^6 cpm was obtained with a particle:hemocyte ratio of 80:1. Higher ratios, 100:1, 120:1, 140:1 and 160:1, resulted in lower peak values, respectively 1.8×10^6 cpm, 2.5×10^6 cpm, 1.96×10^6 cpm and 2.6×10^6 cpm. Lower ratios (from 20:1 to 60:1) resulted in CL responses about 1.5×10^6 cpm.
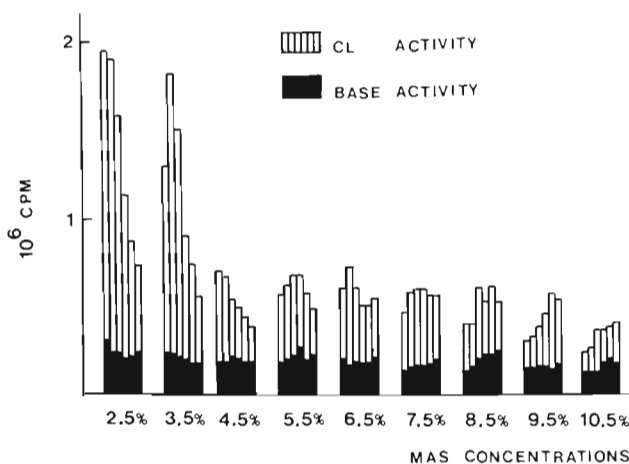

Fig. 1. Chemiluminescence responses of *Crassostrea gigas* hemocytes (10 oyster pool) for different Modified Alsever Solution (MAS) concentrations in the samples. Particle:hemocyte ratio 80:1, luminol concentration 10^{-4} M; CL values recorded at 6 time intervals of 26 min are reported
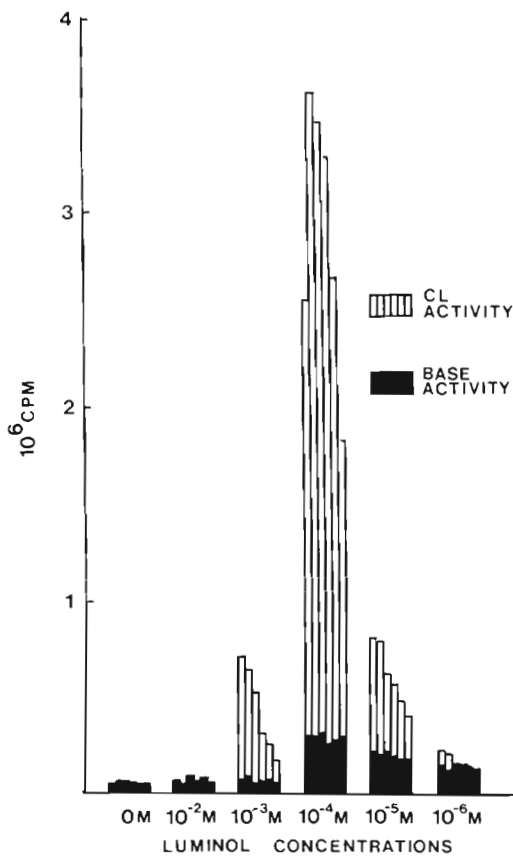
Fig. 2. Effect of luminol concentrations on CL measures of *Crassostrea gigas* hemocytes, pooled from 10 oysters. Particle: hemocyte ratio 80:1. CL values recorded at 6 time intervals of 26 min are reported

CL inhibition assays

Immediately after cytochalasin B treatment, hemocyte preparations showed very strong CL activity (3.24 × 10⁶ cpm) followed by an apparent insensitivity to zymosan stimulation (Fig. 4).

The addition of sodium azide to stimulated hemocytes led to rapid quenching of the CL responses which fell abruptly from 0.92×10^6 cpm to 0.15×10^6 cpm, whereas at the same time, the stimulated uninhibited control sample reached a peak intensity of 1.10×10^6 cpm.

CL assays of individual hemolymph samples of the two oyster species

Individual hemolymph samples were analysed for CL responses after zymosan stimulation (particle:hemocyte ratio 80:1) and the number of stimulated hemocyte was constant (2×10^5) for all individuals. Results are presented in Fig. 5 as the CL responses measured at 7 regular time intervals. These values, obtained for 10 specimens of each species, varied considerably in
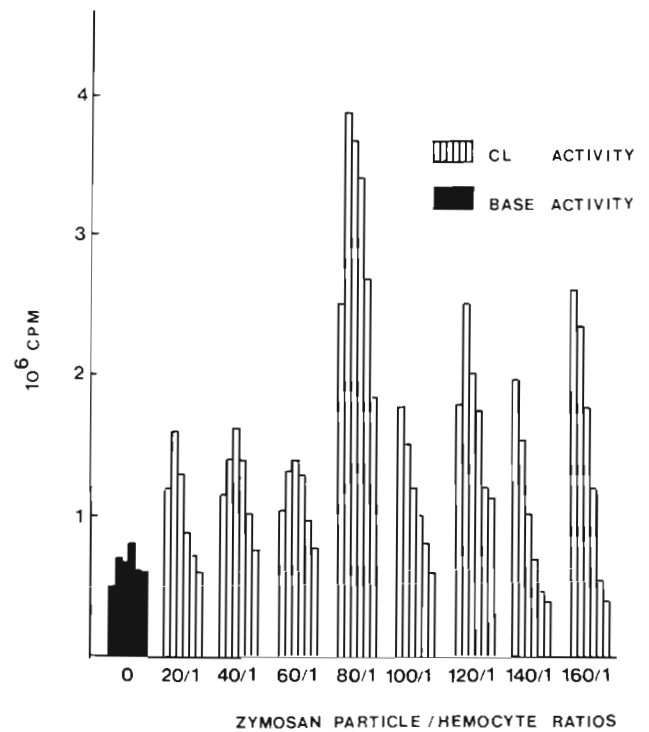

Fig. 3. Chemiluminescence responses of *Crassostrea gigas* hemocytes (10 oyster pool) stimulated by different zymosan particle:hemocyte ratios. CL values recorded for 6 time intervals of 26 min are reported. Luminol concentration 10^{-4} M
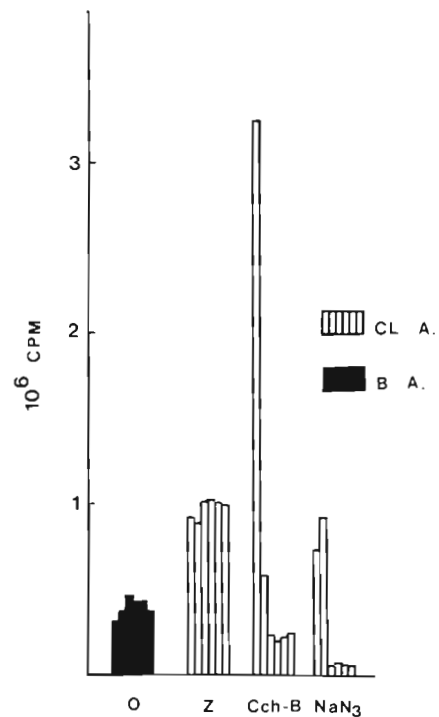

Fig. 4. Effect of cytochalasin B (Cch-B; 1.5 μg ml⁻¹) and sodium azide, NaN₃ (32 μg ml⁻¹) on CL activity of *Crassostrea gigas* hemocytes (10 oyster pool) stimulated by zymosan (80:1). Cch B was added just before zymosan; NaN₃ was added when the cells reached maximum activity. CL values recorded at 6 time intervals of 26 min are reported
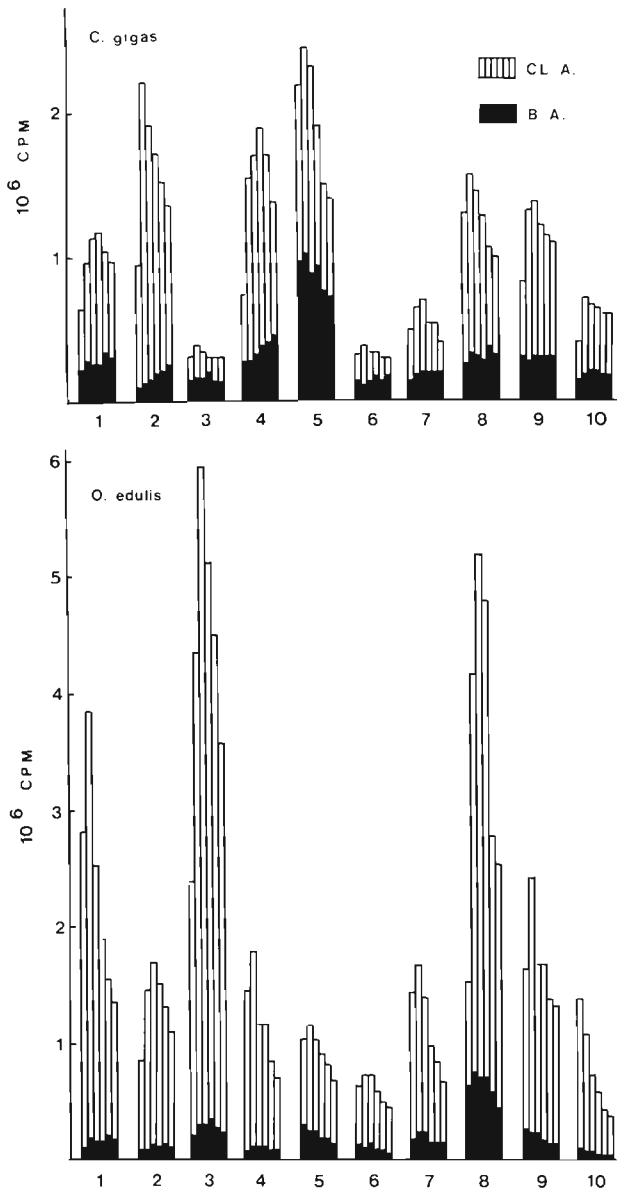
Fig. 5. Chemiluminescence assays on *Ostrea edulis* and *Crassostrea gigas* individual hemolymphs (Individual Nos. 1 to 10). Zymosan particle:hemocyte ratio 80:1. CL values recorded at 6 time intervals of 26 min are reported

intensity and also in swiftness. Taking the peak intensity as a reliable feature (Giltinan et al. 1986) of an individual CL response, comparisons were made between the species. For *Ostrea edulis*, CL values ranged about a mean of 2.59×10^6 cpm ($\pm 1.6 \times 10^6$ cpm) and for *Crassostrea gigas* about 1.28×10^5 cpm ($\pm 0.63 \times 10^6$ cpm) (Table 1). The individual and species CL variability was statistically analysed. Using a correlation analysis, it was shown for both oyster species that the CL activity of the hemocytes was independent of the quantity of MAS present in the samples ($r = 0.15$ for *O. edulis* and $r = 0.62$ for *C. gigas*; df = 8, for $r = 0.63$, the probability is $p = 0.05$).

Since individual variability was established between hemolymph samples with strictly similar hemocyte numbers, the relation between CL peak intensity and the percentage of the different cell types was tested by the estimation of the correlation coefficients r. The results, presented in Table 1, indicate a statistically significant relation between CL response and percentage of granulocytes, and also between CL response and percentage of small hyalinocytes, for both oyster species. Nevertheless, the CL response of *Crassostreas gigas* was high when the percentage of small hyalinocytes (SH) was low ($r = -0.73$) and when the proportion of granulocytes (G) was high ($r = 0.75$). In *Ostrea edulis*, a contrary relation was observed ($r = 0.64$ for the relation between CL response and % SH, and $r = -0.64$ for the relation between CL response and % G). No correlation was found between the CL activity of the oyster hemolymph and the quantity of large hyalinocytes ($r <$ theoretical r).

DISCUSSION

The chief aim of this work was to establish experimental parameters in order to reliably apply the CL technique to the study of phagocytic function and respiratory burst activity of the hemocytes of 2 oyster species, *Ostrea edulis* and *Crassostrea gigas*. This technique was selected from the numerous methods of phagocytosis measurement because CL is considered a valuable and sensitive tool for monitoring the potential activity of phagocyte populations in vertebrates and, therefore, the immunological state of an animal. So we chose CL as the method for quantification of defense responses of individual oysters, looking for a correlation between hemogram features and hemocyte activities.

As also demonstrated in several gastropod species (Dikkeboom et al. 1988b), we showed by the CL technique that oyster hemocytes display metabolic events associated with the phagocytosis of zymosan particles. In practice, the study was not a simple extrapolation of gastropod studies, since the establishment of the CL technique required the definition of several controlled experimental conditions, which had to be adapted to oyster hemocytes.

The first problem encountered with bivalve hemocytes is the rapid aggregation and clumping of the cells. As previously described (Bachère et al. 1988), this difficulty may be greatly reduced by instantaneously diluting the hemolymph, during withdrawal, in MAS, an efficient anti-aggregant. In this way, it is subsequently possible to aliquot hemolymph into several samples which are strictly homogeneous, quantitatively and qualitatively, and consequently well-

Table 1. *Ostrea edulis* and *Crassostrea gigas*. Correlation between chemiluminescence responses of 10 individual oyster hemolymphs, MAS concentrations and hemogram characteristic (cell type percentages) in the respective samples. The peak values obtained for each individual assay, considered as representative of CL activities, were used in correlation tests. G: granulocyte; LH: large hyalinocyte; SH: small hyalinocyte

Ind. no.	O. edulis					C. gigas				
	CL response (10^6 cpm)	MAS conc. (%)	Cell types (%)			CL response (10^6 cpm)	MAS conc. (%)	Cell types (%)		
			G	LH	SH			G	LH	SH
1	3.84	3.1	7	21	72	1.18	2.4	3	85	12
2	1.70	2.5	23	38	39	2.2	2.8	20	62	18
3	5.95	1.1	9	29	62	0.37	1.6	4	69	27
4	1.80	3.1	37	30	33	1.87	2.0	8	79	13
5	1.17	1.9	29	48	23	2.47	1.6	6	79	15
6	0.75	2.1	42	34	24	0.38	1.2	5	67	28
7	1.70	1.4	26	48	26	0.69	1.8	1	72	27
8	5.20	3.1	27	49	24	1.56	2.4	6	77	17
9	2.45	1.6	47	31	22	1.38	3.1	4	79	17
10	1.40	1.8	57	28	15	0.69	1.8	3	75	22
	Mean = 2.59 ± 1.8	r = 0.15	r = − 0.64	r = 0.07	r = 0.64	Mean = 1.28 ± 0.7	r = 0.62	r = 0.75	r = 0.05	r = − 0.73

Theoretical r = 0.63 for df = 8, p = 0.05. Where observed r < theoretical r, there is no significant correlation

adapted for performing statistically comparable assays. This methodology also had the advantage of allowing the cells to be kept in a non-stimulated state. In contrast, hemolymph collected without MAS, or in FSW, led to a high spontaneous CL activity of hemocytes which interfered with their subsequent phagocytic stimulation by zymosan. This CL activity, apparently independent of external stimulation, suggests an auto-activation phenomenon. Contact with the syringe wall or between hemocytes may be sufficient to activate the cells because it leads to perturbation of the hemocyte membrane and probably to depolarization, setting off metabolic events and CL (Van Dyke et al. 1981). This depolarization is dependent on transmembrane movements of Ca^{2+} and Na^+ ions and is prevented by the chelator EDTA, as shown for some vertebrate blood cells which can be kept in a quiescent state in medium without Ca^{2+} until the start of CL assays. However, the phagocytic process needs a sufficient quantity of Ca^{2+} and Mg^{2+}, which must be supplied by the assay medium (Trush et al. 1978, Allen 1986). MAS appeared particularly efficient for oyster hemocytes because it permits passage from a quiescent non-aggregated state to a stimulated sensitive state. This stage change, proved by histological examination of phagocytosis recovery when FSW was added to MAS-diluted hemocyte suspension, was also established by CL provided that the final MAS concentration was about 2.5 % but did not exceed 3.5 %. Thus, MAS/hemolymph (1:1) mixtures need to be diluted 20-fold in FSW to give 2.5 % MAS. Taking into account the range in the

number of hemocytes (1 to 6 × 10^6 hemocytes ml^{-1}) and the hemolymph sample size (about 0.5 ml), several 2 ml aliquots (1 to 3 × 10^5 hemocytes $aliquot^{-1}$) can be obtained from a hemolymph sample. Given that small differences in MAS concentrations (around 2.5 %) did not affect CL, the hemocyte number could be strictly fixed, leading to a comparative aliquoted sample series. In practice, 2 × 10^5 hemocytes per aliquot proved to be adequate to measure a sufficient quantity of CL during a 0.5 min counting period, the assays being performed using luminol as signal enhancer. Indeed, a primary advantage of luminol is to decrease the number of cells required to obtain a significant signal (Allen & Loose 1976). The 10^{-4} M luminol concentration gave the optimal amplification of the CL response whereas at higher concentrations CL responses were reduced or absent, possibly resulting from a toxic effect of the luminol and/or of the DMSO solvent on the hemocytes.

After these experimental parameters had been established, the CL response itself was defined by testing inhibitors of known levels of phagocytic activity. The strong inhibition of CL response by cytochalasin B indicated that CL phenomena were linked to phagocytosis activation of the hemocytes since cytochalasin B interferes with the cytoskeletal movements of the cells. The sudden flash of light observed when adding cytochalasin B may be related to extracellular release of lysosomal enzymes and generation of superoxide ions, O_2^- (Hoffstein et al. 1982). Subsequent contact with zymosan particles, which cannot

be internalized, did not lead to activation of respiratory burst.

When added at CL peak activity, sodium azide had an immediate and strong inhibitory effect on the luminol-enhanced system. Since sodium azide is both a quencher of singlet oxygen $^1O_2$ and a peroxidase inhibitor, and because luminol, as a chemiluminescent bystander molecule, may be an indicator of the myeloperoxidase-H_2O_2-halide system (De Chatelet et al. 1982), it may be inferred that the respiratory burst in bivalves resembles the mechanism in vertebrate phagocytosis. To confirm this hypothesis further research on the biochemical processes of O_2-dependent killing activity in bivalve hemocytes is needed.

As reported for CL assays on the snail *Lymnaea stagnalis* (Dikkeboom et al. 1987), we have shown stimulus-ratio-dependent responses for oyster hemocytes. An optimal zymosan:hemocyte ratio of 80:1 was selected and used in all further experiments. The variable CL responses obtained with the different pooled hemolymph samples during the standardization phase suggested an inter- and intra-specific variability of CL response to the same stimulus. This was confirmed in assays with hemolymph samples from individual oysters (n = 10) containing the same number of hemocytes. The CL responses of *Ostrea edulis* ranged around 2.35×10^6 cpm ($\pm 1.67 \times 10^6$ cpm), whereas for *Crassostrea gigas*, cell activity was 0.97×10^6 cpm ($\pm 0.63 \times 10^6$) cpm. Before conclusions can be made about the significance of these observations, further analyses will be necessary, particularly with oysters collected from different areas and at different times. The reliability of the CL protocol now makes such research possible.

The intraspecific individual CL variability was caused by variability in the granulocyte:small hyalinocyte ratio, although this correlation differed between the 2 oysters. In order to understand the influence of hemogram characteristics on CL responses, the respective involvement of each cell type in CL activity can best be studied with pure hemocyte populations separated using isopycnic and elutriation centrifugation (Bachère et al. 1988).

In the present poor state of knowledge on molluscan defense mechanisms, the CL technique could be particularly suitable for analysing the immunological state of oysters. In studying immune responses against pathogens or pollutants (Wishkovsky 1988), it will be possible to investigate stressor-hemocyte interactions in vitro, to follow defense activity in time for individual oysters and to gain understanding of the development or remission of pathological processes. From a more applied point of view, CL could also be considered as an interesting marker to test for individual genetic selection. With the recent development of hemocyte separation protocols

(Bachère et al. 1988) and of native pathogen purification (Mialhe et al. 1988), such a technique appeared necessary for investigating the killing mechanisms of bivalves hemocytes, and for comparative studies on antimicrobial defense capacity between individuals. Moreover, from a parasitological point of view, research could be undertaken on the interactions of intrahemocytic parasites such as *Bonamia ostreae* with their hosts, in order to understand and to compare their survival mechanisms with those of vertebrate macrophage parasites such as *Leishmania* (Alexander & Russel 1985), *Toxoplasma* and *Trypanosoma* (Moulder 1985, Zingales & Colli 1985).

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