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In vitro modulation of reactive oxygen and nitrogen intermediate (ROI/RNI) production in Crassostrea gigas hemocytes

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Abstract:

Bivalve hemocyte competence has been measured by quantifying functional characteristics, including reactive oxygen intermediate (ROI) production after activation with zymosan or phorbol myristate acetate (PMA). However, untreated oyster hemocytes also produce ROI and RNI (reactive nitrogen intermediates) after bleeding even if not stimulated by zymosan or PMA. Extensive investigation of this parameter by flow cytometry showed that, *in vitro*, ROI/RNI production by untreated hemocytes maintained in seawater appeared to be independent of both bacterial burden in the serum and non-self particle phagocytosis. ROI/RNI production in granulocytes was higher than in hyalinocytes and could be intensified when activated by zymosan but not by PMA. Both cell types used NADPH-oxidase- and NO-synthase-like pathways to produce these molecules; the NO-synthase pathway seemed relatively more dominant in hyalinocytes and NADPH-oxidase appeared more effective in granulocytes. These results provide new insights for interpreting the modulation of ROI/RNI production by untreated hemocytes shown by other studies, relative to environmental conditions or physiological status of the oysters.

Keywords: *Crassostrea gigas*; Hemocytes; Reactive oxygen intermediate (ROI); Reactive nitrogen intermediate (RNI); Flow cytometry; NADPH-oxidase; NO-synthase

1. Introduction

Reactive oxygen intermediate (ROI) production by bivalve hemocytes after stimulation by non-self particles is involved in internal defense responses of bivalves (Cheng, 1996, 2000; Chu, 2000). Production of ROI, after activation by phorbol myristate acetate (PMA) (Nakayama and Maruyama, 1998; Arumugam et al., 2000a; Takahashi and Mori, 2000; Goedken and De Guise, 2004) or during phagocytosis of zymosan particles (Bachère et al., 1991; Le Gall et al., 1991; Bramble and Anderson, 1998; Lambert and Nicolas, 1998; Lambert et al., 2001), has been used frequently to estimate defense competence of bivalve hemocytes. Involvement of an NADPH-oxidase pathway in the production of ROI, superoxide anion and H_2O_2 by bivalve hemocytes has been shown (Noël et al., 1993; Torreilles et al., 1996; Winston et al., 1996; Arumugam et al., 2000b; Takahashi and Mori, 2000). Likewise, a nitric oxide (NO) synthase pathway leading to nitric oxide and peroxynitrite (RNI) synthesis has been suggested (Arumugam et al., 2000b; Gourdon et al., 2001; Torreilles and Romestand, 2001). Various methods have been employed to detect ROI production in hemocytes after activation by zymosan or PMA, either by using anti-aggregant solutions, such as Modified Alsevier Solution (MAS) (Bachère et al., 1991; Le Gall et al., 1991; Lambert and Nicolas, 1998; Lambert et al., 2001) or buffers containing glucose (Bramble and Anderson, 1997; Bramble and Anderson, 1998; Takahashi and Mori, 2000), both known as inhibitors of hemocyte ROI production (Torreilles et al., 1999). Several studies have described such ROI production in bivalve hemocytes without additional stimulation: similar levels of superoxide anion production (measured by NBT reduction) were observed in Crassotrea virginica hemocytes challenged with yeast and in non-challenged hemocytes (Fisher et al., 1996) . Similar observations were done in Crassostrea gigas hemocytes by using a flow-cytometric (FCM) method based upon the intracellular oxidation of 2',7'-dichlorofluorescin (DCFH) into green-fluorescent dichlorofluorescein (DCF) to measure production of ROI/RNI by the two main hemocyte sub-populations in oysters: granulocytes and hyalinocytes (Lambert et al., 2003). Indeed, this study showed not only that ROI/RNI production can be measured in hemocytes simply maintained in filtered sterile seawater (FSSW) after bleeding, but also that this production is higher in granulocytes than in hyalinocytes. Knowledge of the specific role of the two main C. gigas hemocyte sub-populations - granulocytes and hyalinocytes - in the synthesis of ROI/RNI, however, is scarce. Moreover, the respective contribution of ROI and RNI production in DCFH oxidation by granulocytes and hyalinocytes is unknown.

Thus, several basic questions regarding ROI/RNI production by oyster hemocytes remained unanswered: 1) what are the causes of ROI and RNI production in untreated hemocytes, i.e. without additional activation? 2) Are the pathways involved in ROI/RNI production by untreated granulocytes and hyalinocytes similar?

To address these questions, the present study applied FCM, coupled to DCFH oxidation, to test various modulators on the ROI/RNI production by two main C. gigas hemocyte sub-populations (granulocytes and hyalinocytes) maintained in sea water after bleeding.

2. Materials and methods

2.1. ROI/RNI production assays

2.1.1. Animals

Adult oysters (ca. 2 yr old) from Brittany (France) were transported to the laboratory at the IUEM (Institut Universitaire Européen de la Mer, Brittany). After arrival, animals were acclimated for at least 24 hours in a flow-through seawater system at 10-13°C temperature, 33-35 ‰ salinity, and then maintained in these conditions for a maximum of one week before hemolymph extraction.

2.1.2. Hemolymph collection

Hemolymph was withdrawn from the adductor muscle using a 1- or 2-mL plastic syringe fitted with a 25-gauge needle through a notch previously ground in the shell. Hemolymph from each oyster was transferred into an individual micro-tube held on ice. Individual hemolymph samples were examined under the microscope to eliminate samples with contaminating particles, such as gametes or tissue debris. Hemolymph from at least 5 animals was mixed to constitute each pool. Four pools from at least 20 oysters were made for each experiment, except as mentioned. Then, hemolymph was filtered through a 80-µm mesh before FCM analysis to avoid clogging of the flow cytometer by un-detected debris.

2.1.3. Reactive oxygen and nitrogen intermediate (ROI/RNI) production

The method for measuring ROI/RNI production was adapted from previous works (Lambert et al., 2003). Briefly, sub-samples of hemolymph from each of the four pools were distributed into 5-ml polystyrene tubes (Falcon®, B-D Biosciences, San Jose, CA, USA) and maintained on ice. 2'7'-dichlorofluorescin diacetate (DCFH-DA, Sigma) was added to yield a final concentration of 10 µM. Flow-cytometric measurement, using a BD BioSciences FACSCalibur flow-cytometer, with 0.2-µm filtered PBS azide as sheath fluid (NaCl 0.14 M, KH_2PO_4 anhydrous 10 mM, Na_2HPO_4 anhydrous 56.8 mM, di-sodium EDTA 3.4 mM, NaN_3 1.54 mM), was then performed at t = 0, 60, 120 and 180 minutes. Between measurements, the tubes were maintained at room temperature (20/ 22°C).

Flow-cytometric measures of DCF green fluorescence, related quantitatively to hemocyte ROI/RNI production, at each incubation time, and for each hemocyte sub-population (small agranulocytes, hyalinocytes and granulocytes), were recorded on the FL1 detector and expressed as arbitrary units (method described in (Lambert et al., 2003). DCFH can be oxidized into fluorescent DCF by ROIs, H_2O_2 (Bass et al., 1983), superoxide anion but also nitric oxide (NO) (Rao et al., 1992; Curtin et al., 2002). To simplify presentation, except if noted, only results for hyalinocytes and granulocytes obtained after 120 min of incubation are presented.

2.1.4. Toxicity

The potential toxicity of solvents or modulators used was tested by evaluating hemocyte viability in an anti-aggregant solution (AASH) developed for bivalve hemocytes (Auffret and Oubella, 1994), at a 1/1 vol/vol dilution with hemolymph after 180 min incubation at room temperature. Viability of C. gigas hemocytes was evaluated by FCM, using a double staining procedure (SYBR®Green and propidium iodide, PI, Sigma). AASH was used to reduce the aggregation of cells which can lead to an over-estimation of the number of dead cells as aggregates containing at least one dead cell (PI-stained) surrounded by numerous viable cells were counted as “dead”. AASH has been shown to have no effect on C. gigas hemocyte viability (data not shown). Results are presented as percentages of dead hemocytes after 180 min incubation with appropriate concentrations of modulator (Cf. Table 1: IAA, NMMA, DPI, PMA) and compared to control.

2.1.5. ROI/RNI production by untreated hemocytes

The level of ROI/RNI production of untreated granulocytes and hyalinocytes was compared after 120 min incubation at room temperature in FSSW, from 5 experiments.

2.1.6. Bacterial burden

To explore the possible effect of bacteria present in the hemolymph on the observed ROI/RNI production in hemocytes when maintained in seawater, the possible relationship between bacterial burden in serum and hemocyte ROI/RNI production, after 120 min of incubation in sea water, was explored. 20 oysters were bled individually, and a tetrazolium dye-reduction assay was applied to serum (after hemocytes were removed by centrifugation), to estimate bacterial cell concentration. The assay used was modified from previous methods (Volety et al., 1999). Results are expressed in units of optical density at 492 nm, which detects the enzymatic reduction of the tetrazolium dye by living bacterial cells, providing estimation of the concentration of alive bacteria. In parallel, granulocyte and hyalinocyte ROI/RNI production was evaluated, as described above but for individual oysters rather than pools.

2.1.7. Total hemocyte counts

Hemocyte concentrations in pools were evaluated to adjust the ratios of hemocytes/ zymosan particles when needed. One 100-µL sub-sample from each pool was fixed by adding a formalin solution of 4% in FSSW. After 30 minutes incubation at room temperature (20/22°C) with the SYBR® Green (10X final concentrations), samples were then analyzed on the flow cytometer. A density plot visualization of side scatter (SSC) vs. FL1 permitted differentiation and gating of hemocytes stained by SYBR® Green from other particles in the hemolymph. The flow rate of the cytometer was measured for each experiment as described previously (Marie et al., 1999): briefly, a tube containing distilled water was

weighed before and after a 10 min flow cytometer run to determine the volume analyzed over a known time in µL min^{-1}; this value was used to calculate the hemocyte concentration in cells mL^{-1}.

2.1.8. Hemocyte response to modulators

Granulocyte and hyalinocyte ROI/RNI production was measured after 120 min incubation with the modulators (activators or inhibitors) presented in Table 1 and also with addition of both zymosan and cytochalasin B.

2.1.9. Statistical analysis

Significant differences between conditions during each assay were tested, using the STATGRAPHICS Plus 5.1 software, by ANOVA, or the Kruskall-Wallis test in cases wherein variance was not normal. The method used to discriminate differences between means was Fisher's least significant difference (LSD) procedure. Differences were considered significant at $p < 0.05$. Possible relationships between bacterial burden and ROI/RNI production in granulocytes or in hyalinocytes were tested with the STATGRAPHICS Plus 5.1 software by calculating the R^2 value for linear and polynomial regression functions relating these two variables.

3. RESULTS

3.1. ROI/RNI production assays

3.1.1. Toxicity

Across all assays, mean percent mortality of hemocytes maintained for 180 min in FSSW ranged from 1.8 to 7.2 %. No significant differences were observed for hemocytes exposed to the following treatments: IAA 1 and 10 mM, NMMA 50 and 500 µM, DPI 5 and 50µM, PMA 1 and 10 µg mL^{-1}, leading to conclude that these chemicals were not affecting hemocyte viability in these conditions.

3.1.2. ROI/RNI production by untreated hemocytes

As shown in Fig. 1, from 60 to 180 min, increases in intracellular fluorescence indicated production of ROI/RNI. The maximal increase in ROI/RNI (slope) was found during the first 60 min, but the increase continued during the second time period. Hyalinocytes showed a significantly-lower ROI/RNI production than granulocytes for all incubation times in FSSW (ANOVA, $p< 0.05$).

3.1.3. Bacterial burden

Bacteria were found at various concentrations in individual oyster serum pools as indicated by tetrazolium dye reduction assay, from 0.01 to 0.35 unit DO_{492nm} (Fig.2). The R^2 values for all regression functions tested, relating hyalinocyte or granulocyte ROI/RNI production and serum bacterial burden were consistently less than 0.1 ($P = 0.71$ to 0.98).

3.1.4. Hemocyte response to activators

Zymosan: ROI/RNI production, measured in both granulocytes and hyalinocytes during phagocytosis of zymosan particles, is presented in Table 2. It is clear that granulocytes were able to produce extra ROI/RNI when challenged with zymosan, as compared to the level obtained with untreated granulocytes (ANOVA, $p<0.01$, $n=19$). By contrast, no significant zymosan-induced modification in ROI/RNI production by hyalinocytes was observed.
PMA: Addition of PMA at 1 µg mL^{-1} did not modify the ROI/RNI production of either granulocytes or hyalinocytes, as compared to the level observed in untreated hemocytes (Table 2). However, when added at 10 µg mL^{-1}, PMA significantly reduced ROI/RNI production of all hemocyte types.

3.1.5. Hemocyte response to inhibitors

DPI: ROI/RNI production was 62 % and 72% lower with DPI 5 µM and 82% and 84% lower with DPI 50 µM, for granulocytes and hyalinocytes, respectively, compared to hemocytes without DPI (Table 2).
IAA: For hyalinocytes, no significant differences were observed in ROI/RNI production with or without IAA (Table 2), regardless of doses (1 or 10 mM final concentration). However, production of ROI/RNI by granulocytes was significantly lowered after 120 min incubation in IAA, and decreased by 70%, for both IAA doses (1 and 10 mM).
NMMA: For hyalinocytes, production of ROI/RNI was significantly lower in the presence of NMMA (50 and 500 µM: ANOVA, $p<0.05$) and decreased by 27% and 33%, respectively compared without NMMA. For granulocytes, addition of NMMA did not cause any significant modification of ROI/RNI production (Table 2).
Cyt B: In the presence of cytochalasin B at 10 µg mL^{-1}, no significant modification of ROI/RNI production was observed for granulocytes, compared without Cyt B (Fig. 3). The same result was obtained with hyalinocytes.
Zymosan + Cyt B: Addition of zymosan, as shown previously, increased the ROI/RNI production in granulocytes, but combined with Cyt B, the production is reduced to the level observed in the control (Fig. 3). The same result was obtained with hyalinocytes.

4. DISCUSSION

Recent observations (Lambert et al., 2003) of the capacity of oyster hemocytes to produce ROI/RNI, even when simply maintained in sterile seawater after bleeding, prompted the present study. Thus, two basic questions regarding ROI/RNI production by oyster hemocytes have been addressed in this study 1) what are the causes of ROI and RNI production in untreated hemocytes, i.e. without additional activation? 2) Are the pathways involved in ROI/RNI production by untreated granulocytes and hyalinocytes similar?

1) The initial indication of the causes of ROI and RNI production in untreated hemocytes was obtained by using DPI, a strong inhibitor of both NADPH-oxidase and NO-synthase pathways (Li et al., 2003). Approximately 80 to 85% of the fluorescence measured on untreated C. gigas hemocytes was attributable to ROI/RNI produced by enzymatic systems usually described during the "respiratory burst" phenomenon. The 15 to 20% remaining could correspond to a normal, constitutive physiological production of oxidant molecules. Cell metabolism, particularly within mitochondria, is known to produce ROI (Poderoso et al., 1996; Valdez et al., 2000; Marchetti et al., 2002; Cadenas, 2004), and it is likely that these could oxidize the intracellular DCFH into fluorescent DCF. For the main portion of the fluorescence measured, several hypotheses can be proposed. First, the presence of bacteria in oyster hemolymph could stimulate hemocyte ROI/RNI production, as well as phagocytosis of non-self material. Indeed, bacteria can be present in hemolymph, even in healthy marine invertebrate (Sizemore et al., 1975; Tubiash et al., 1975), and especially healthy oysters (Olafsen et al., 1992; Garnier et al., in press). However, in the present study, no relationship was found between bacterial burden in the serum and ROI/RNI production. Moreover, cytochalasin B did not modify the ROI/RNI production in untreated hemocytes, while it did significantly reduce the extra ROI/RNI induced by phagocytosis of zymosan. Cytochalasin B is known to inhibit the actin polymerization implicated in phagocytosis. These two results suggest that the ROI/RNI production by untreated C. gigas hemocytes was not directly or simply linked to phagocytosis of bacteria and/or non-self material.
A second hypothesis to explain the ROI/RNI production of untreated hemocytes may rely on the stimulation of the hemocyte membrane following contact of cells with non-self materials (e.g., needle, syringe, plastic tubes) as observed on mussels (Pipe, 1992) or hypothesized for crustacean hemocytes (Bachere et al., 1995; Moss and Allam, 2006). However, recent works have shown that ROI/RNI production by untreated hemocytes from marine invertebrates can be modulated according to various environmental factors like food availability or algal diets in C. gigas (Delaporte et al., 2006a; Delaporte et al., 2006b) , in vitro and in vivo infection by Vibrio aestuarianus in C. gigas (Labreuche et al., 2006b; Labreuche et al., 2006a) or level of dissolved oxygen in rearing sites in lobster *Homarus americanus* (Moss and Allam, 2006). In-depth comprehension of such a modulation is still at an early stage but it seems important to add ROI/RNI production by untreated hemocytes as a one of the indicator of the oyster fitness.
During this study, PMA did not show any capacity to modify ROI/RNI production in both untreated C. gigas hemocyte types: granulocytes and hyalinocytes. PMA activates intracellular protein kinase C (Li et al., 2000), catalyzing the phosphorylation of the cytosolic proteins in the NADPH-oxidase complex (Torreilles et al., 1996) following their migration to the membrane proteins of the complex that finally become activated. The absence of response in this study confirmed previous results (Delaporte et al., 2003; Lambert et al., 2003). These authors hypothesized that the level of ROI/RNI production by untreated hemocytes could be high enough to make PMA addition ineffective. Same kind of observations were done recently on crustacean hemocytes by others authors (Moss and Allam, 2006). This, however, could appear contradictory, particularly to the findings of works done on C. gigas hemocyte ROI production (Nakayama and Maruyama, 1998). They showed that superoxide anion production measured by MCLA-dependent chemiluminescence, was activated by PMA. This apparent contradiction could be explained, among others, by the fact that during bleeding, these authors kept hemocytes in pure anti aggregant solution (modified Alsever solution, MAS). MAS is well known to strongly reduce ROI production in bivalve hemocytes (Torreilles et al., 1999) and to let cells in a rested state, producing no or almost no ROIs. Before their superoxide assay, the authors removed the anti-aggregant solution, and PMA was then able to activate hemocyte ROI production.

2) Progress in further understanding the specific roles of hyalinocytes and granulocytes in ROI/RNI production when untreated was done in the present study by using an original FCM method. As a result, granulocytes were shown to produce appreciably higher level of ROI/RNI than hyalinocytes. Moreover, when activated by phagocytosis of zymosan particles, only granulocytes were able to produce extra ROI/RNI, at least in a systematically significant manner. These two results could

indicate that the two main C. gigas hemocyte sub-populations possess different capabilities for ROI/RNI response. In the same manner, differences were also observed in C. virginica: ROI/RNI production in granulocytes and intermediate cells can be activated (PMA), but hyalinocytes cannot (Goedken and De Guise, 2004). Consequently, measuring the proportion as well as the concentration of hemocyte sub-populations become of major importance to evaluate the functional capacity of hemocytes.
We also tested in this study specific inhibitors to better understand the respective roles of each pathway in the ROI/RNI synthesis of the two hemocyte types. Contrary to DPI findings, these two inhibitors had different effects on the granulocyte and hyalinocyte sub-populations. The IAA drastically reduced granulocyte ROI/RNI production (71%) when it was not significant in hyalinocyte. By contrast, NMMA was a more potent inhibitor of hyalinocyte ROI/RNI production (27 to 33% decreases) and had no significant effect on granulocytes. To the best of our knowledge, this is the first report of different responses to an inhibitor according to the hemocyte cell type in C. gigas showing preferential NADPH-oxidase pathway for granulocyte ROI synthesis and NO-synthase pathway for hyalinocytes RNI production. The contribution of a NO-synthase pathway to "phagocytosis-associated ROI production" in C. gigas have been previously proposed (Nakayama and Maruyama, 1998; Torreilles and Romestand, 2001), but the present study show that the NO-synthase pathway is more active in hyalinocytes. Some other functional differences according to C. gigas hemocytes cell type have been recently suggested (Terahara et al., 2006), saying that in C. gigas "phagocytosis of hyalinocytes is regulated by an integrin-dependent mechanism and that of granulocytes is elicited by other functional receptors". All these results highlight the specificity of the two main hemocyte cell types in C. gigas, according to their functional capacities and may provide new insights in the interpretation of the modulation of the hemocyte parameters according to environmental conditions or physiological status of the oysters.

As a conclusion, this study provided a better understanding of the ROI/RNI production by untreated C. gigas hemocytes, measured in vitro in seawater after bleeding. This ROI/RNI production has been shown to be independent of bacterial burden in the serum or from phagocytosis of non-self particles. The two main C. gigas hemocyte cell types, granulocytes and hyalinocytes, have been shown to possess different ROI/RNI production capacities, granulocytes being the most active in terms of constitutive production and capacity to produce extra ROI/RNI during phagocytosis of zymosan particles. This study showed that both cell types used NADPH-oxidase- and NO-synthase-like pathways to produce ROI/RNI but the NO-synthase pathway seemed more dominant in hyalinocytes while NADPH-oxidase was more effective in granulocytes. Finally, it seems interesting to go further in the survey of such a parameter, especially by looking at hemocyte sub-populations, to better understand the oyster fitness or the impact of environmental conditions.

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Table 1: list and concentration of modulators tested on ROI/RNI production by untreated C. gigas hemocytes.

Modulator	chemicals	Final Concentration	Activity	Ref.
Activator	Zymosan	20 particles hemocyte^{-1}	Hemocyte phagocytosis, ROI/RNI production	1.2.3. 4.5.6
	Phorbol myristate acetate (PMA)	1 and 10 µg mL^{-1}	Activation of the NADPH-oxidase complex	7.8
inhibitor	Diphenylene iodonium chloride (DPI)	5 and 50 µM	NADPH-oxidase and NO-synthase inhibitor	9.10
	iodoacetamide acetate salt (IAA)	1 and 10 mM	NADPH-oxidase inhibitor	11
	NG MonoMethyl-L-Arginine monoacetate (NMMA)	50 and 500 µM	NO-synthase inhibitor	12
	Cytochalasin B	10 µg mL^{-1}	phagocytosis inhibitor	1.2

1: (Bachère et al., 1991) ; **2**: (Le Gall et al., 1991) ; **3**: (Bramble and Anderson, 1998) ; **4**: (Lambert and Nicolas, 1998) ; **5**: (Lambert et al., 2001) ; **6**: (Lambert et al., 2003) ; **7**: (Torreilles et al., 1996) ; **8**: (Li et al., 2000) ; **9**: (Bramble and Anderson, 1997) ; **10**: (Torreilles and Romestand, 2001); **11**: (Pipe, 1992); **12**: (Arumugam et al., 2000a).

Table 2: Mean ROI/RNI (reactive oxygen/ nitrogen intermediates) production (DCF fluorescence, FL1 value, in arbitrary unit, n=4, except for zymosan assay n=19, ± standard error, SE) of C. gigas hemocytes (granulocytes and hyalinocytes) after 120 min incubation in filtered sterile sea-water (untreated hemocyte = control) or with various modulators. The table group together results obtained from different experiments (*: indicate significant difference with the appropriate control, ANOVA or Kruskall-Wallis, $p < 0.05$).

	Granulocytes (mean ± SE)	**Hyalinocytes** (mean ± SE)
Control (untreated)	**190 ± 18**	**100 ± 13**
+ zymosan	275 ± 25 *****	106 ± 11
Control (untreated)	**210 ± 28**	**107 ± 11**
+ PMA 1 µg mL^{-1}	167 ± 14	83 ± 10
+ PMA 10 µg mL^{-1}	138 ± 13 *****	69 ± 8 *****
Control (untreated)	**276 ± 51**	**165 ± 40**
+ DPI 5 µM	105 ± 19 *****	46 ± 7 *****
+ DPI 50 µM	49 ± 11 *****	26 ± 5 *****
Control (untreated)	**162 ± 28**	**82 ± 31**
+ IAA 1 mM	51 ± 11 *****	29 ± 12
+ IAA 10 mM	47 ± 15 *****	36 ± 13
Control (untreated)	**227 ± 21**	**125 ± 8**
+ NMMA 50 µM	190 ± 25	91 ± 10 *****
+ NMMA 500 µM	193 ± 20	84 ± 6 *****

FSSW: filtered sterile sea-water.
Zymosan: 20 particles $hemocyte^{-1}$.
PMA: phorbol myristate acetate.
DPI: diphenylene iodonium chloride.
NMMA: NG MonoMethyl-L-Arginine monoacetate.
IAA: iodoacetamide acetate salt.

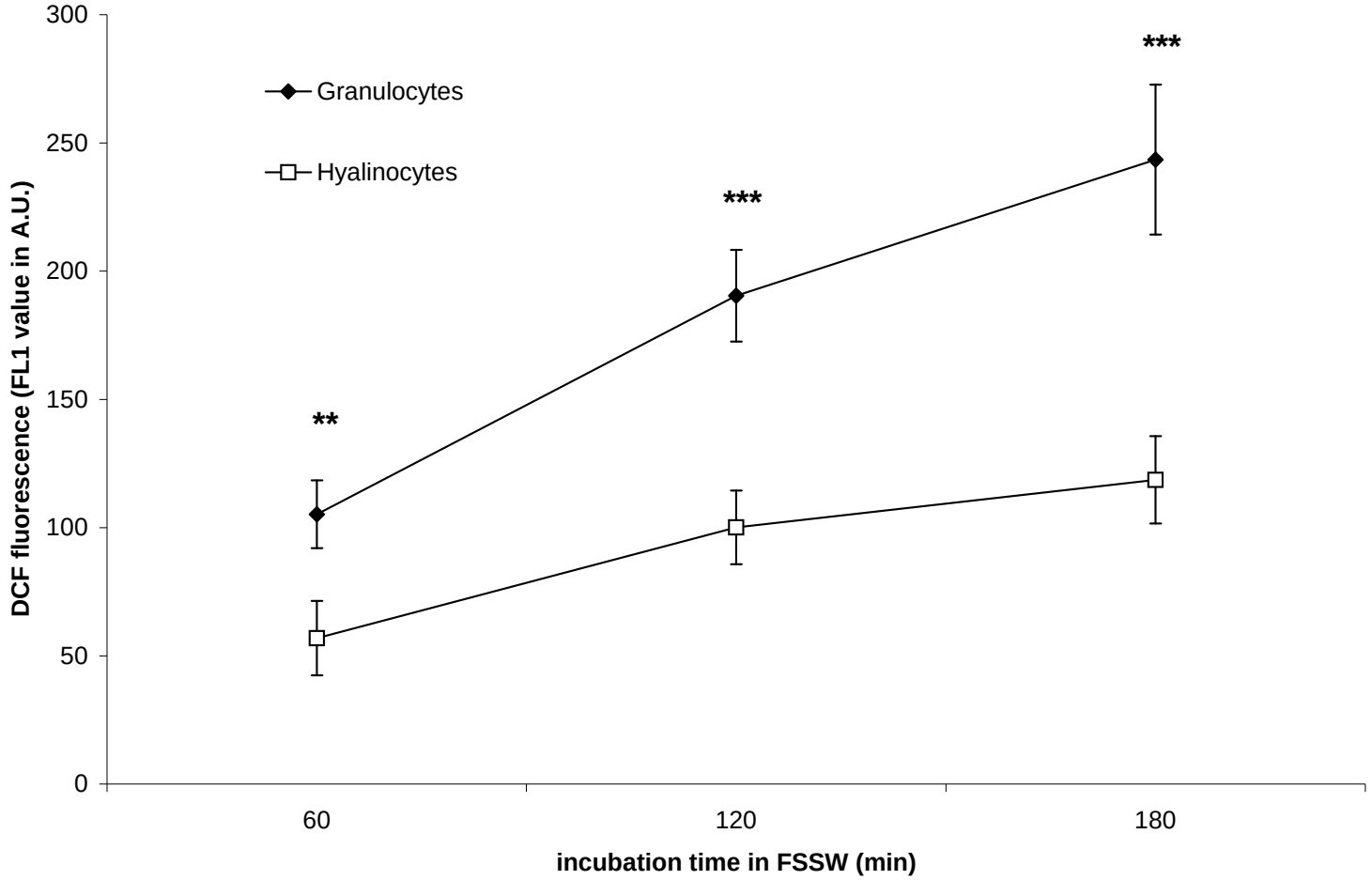

Fig.1: Comparison of the mean level of ROI/RNI production expressed as DCF fluorescence (FL1 value, in arbitrary unit A.U., n=19 + SE) in C. gigas granulocytes and hyalinocytes at different time of incubation (60, 120, 180 min) in FSSW at ambient temperature. (mean comparison: ANOVA; NS: no significant differences, ** significant differences at $p<0.01$, *** $p<0.001$).

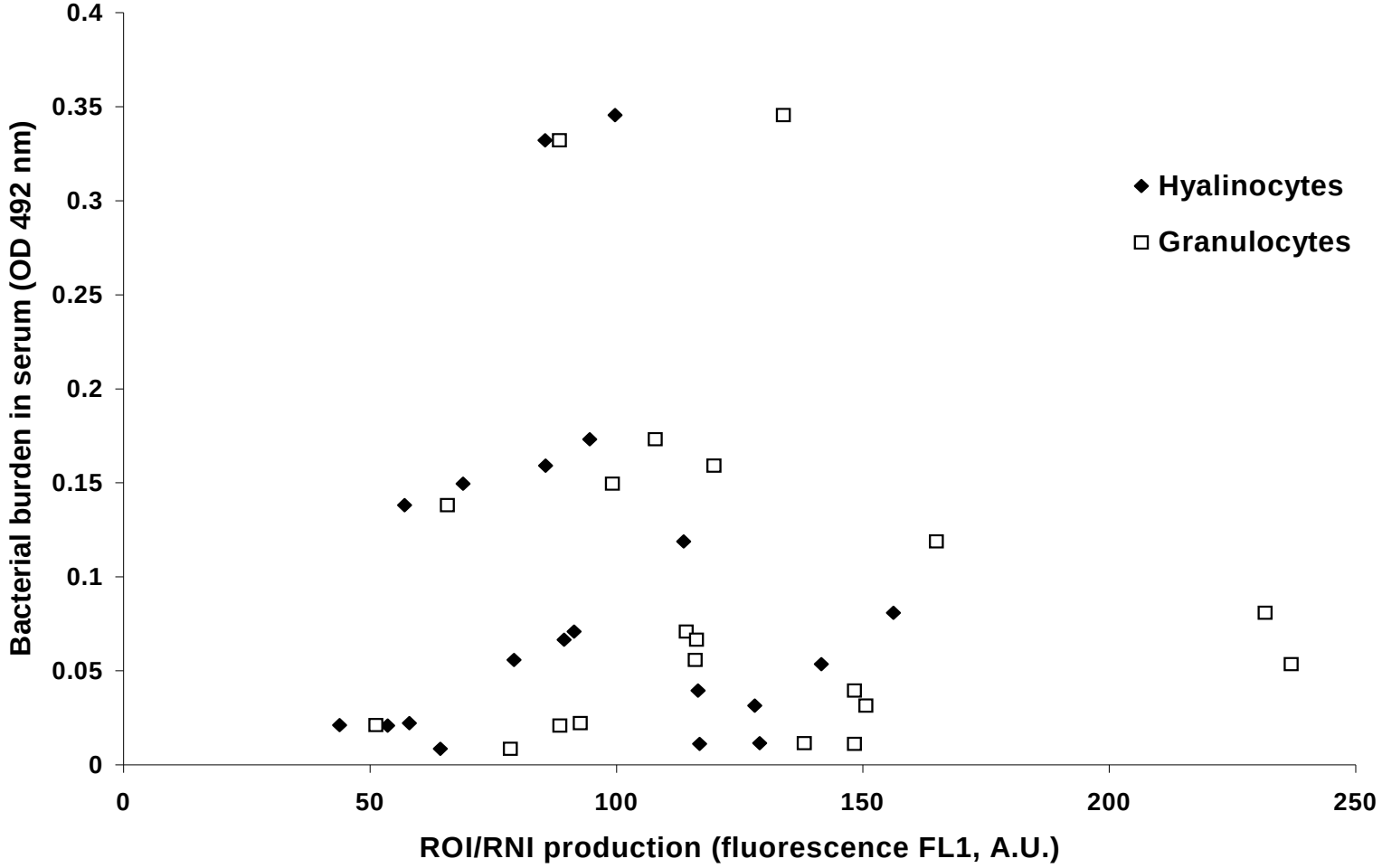

Fig.2: Individual level of ROI/RNI production expressed as DCF fluorescence (FL1 value, in arbitrary unit A.U.) by untreated granulocytes and hyalinocytes from twenty C. gigas after 120 min incubation at ambient temperature in FSSW according to the bacterial burden in serum evaluated by using a tetrazolium dye reduction assay (OD 492 nm).

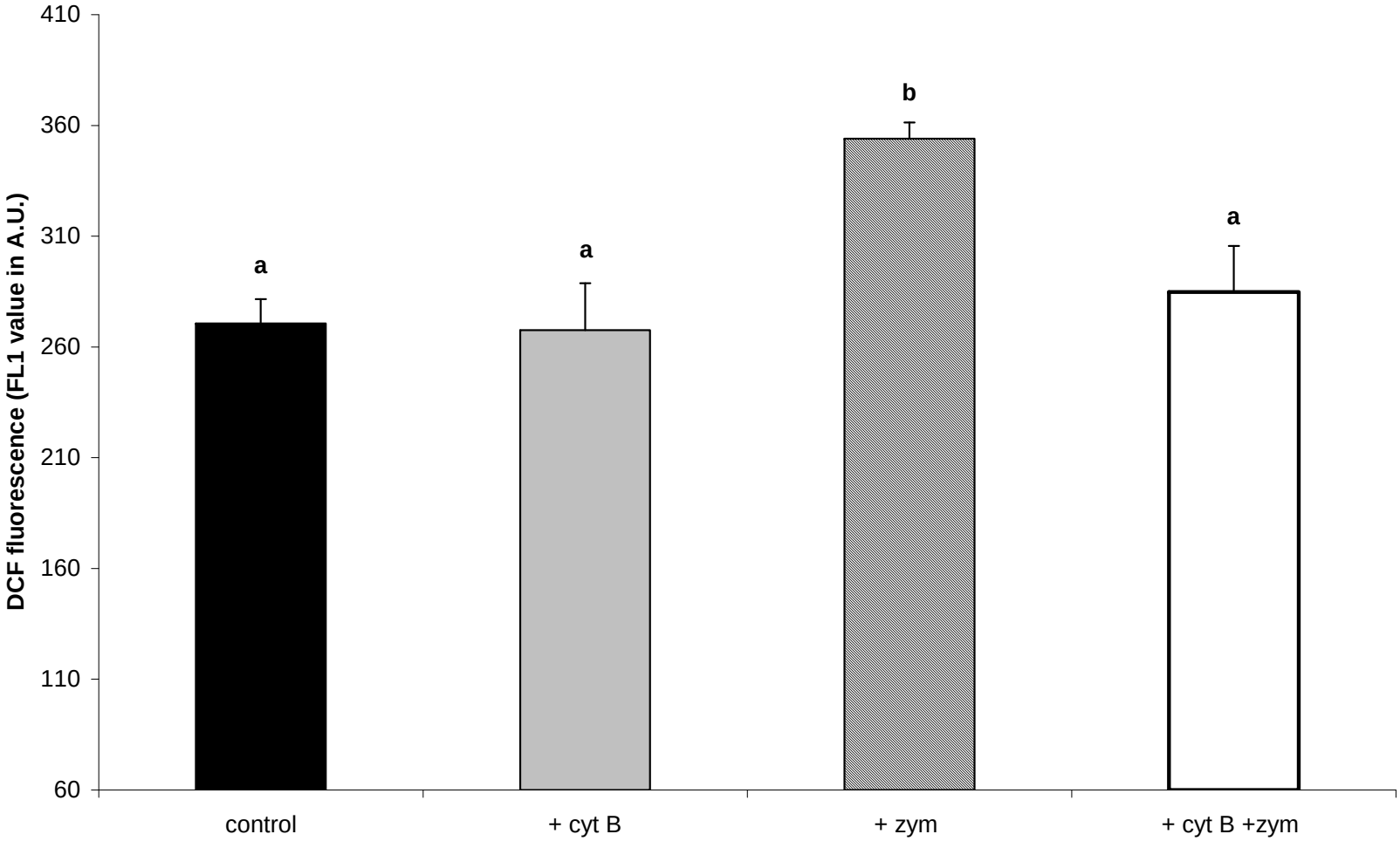

Fig.3: Comparison of the mean level of ROI/RNI production expressed as DCF fluorescence (FL1 value, in arbitrary unit A.U., n=4 + SE) in C. gigas granulocytes at 120 min incubation at ambient temperature in FSSW (control) or after addition of cytochalasin B (**+ cyt B**: 10µg mL^{-1} final concentration), zymosan (**+ zym**: 20 particles $hemocyte^{-1}$) or both chemicals (**+ cyt B + zym**). (Letters indicates significant differences between conditions, ANOVA, $p< 0.05$).