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Fluorimetric determination of hydrogen peroxide production by the haemocytes of the wax moth *Galleria mellonella* (Lepidoptera: Pyralidae)

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Abstract. The aim of this study was to investigate whether haemocytes of *Galleria mellonella* (Lepidoptera: Pyralidae) larvae produce reactive oxygen species (ROS) like human blood phagocytes. The production of ROS was measured first using luminol-enhanced chemiluminescence of un-stimulated and stimulated (four activators with different modes of action) haemolymph or isolated haemocytes. However, spontaneous and activated production of ROS remained at the background level. In subsequent experiments an ultrasensitive fluorescence method using Amplex Red reagent to detect hydrogen peroxide (H_2O_2) was used. After optimization, Amplex Red was successfully used for determining H_2O_2 production by both un-stimulated and stimulated haemocytes. To determine the affect of pH and ions on the measurement, several diluent solutions were tested. This revealed that Ca^{2+} and Mg^{2+} ions are less important for the reaction in insect than mammalian cells. Among the activators tested, phorbol myristate acetate (PMA) and calcium ionophore (Ca-I) had the best stimulatory effect on insect samples, while opsonised zymosan particles (OZP) was the best activator for human phagocytes. In conclusion, the haemocytes of *G. mellonella* produce H_2O_2 as an important innate immunity factor, but under different conditions and in different amounts, which probably results in them being less effective in killing microbes than human phagocytes.

INTRODUCTION

The immune responses of insects to invading pathogens can be divided into cellular (also called hemocytic) and humoral. Both protect insects against invaders that have overcome the physical barriers posed by the insects' integument, mid-gut epithelium and peritrophic membrane (Elrod-Erickson et al., 2000). Cellular responses, characterized by a direct interaction of haemocytes with antigens, involve phagocytosis, microaggregation, nodulation and encapsulation. Humoral defence involves the action of molecules constitutively present in tissues and haemolymph, such as lectins or lysozymes, induced synthesis of antimicrobial peptides (AMPs) and activation of the phenoloxidase (PO) cascade, while coagulation cascades involve both cellular and humoral mechanisms (Theopold et al., 2002). The insect immune response has a number of structural and functional similarities to the innate immune response of mammals (e.g. Vilmos & Kurucz, 1998; Salzet 2001; Kavanagh & Reeves, 2004, 2007; Wang et al., 2010). The mechanism and probably also the proteins involved in the production of superoxide in insect haemocytes are similar to those involved in the NADPH oxidase-induced superoxide production in human neutrophils (Bergin et al., 2005). Insect haemolymph contains haemocytes, which function in a manner similar to that of the phagocytes of humans (Ratcliffe, 1993). At least six types of haemocytes are present in insects (Boman & Hultmark, 1987), and plasmatocytes and granulocytes are the most abundant types of phagocytic cells. Production of ROS, as an important tool for oxidative killing of invading pathogens during phagocytosis, has been detected in some insects, other invertebrates and mammals; there is also evidence that both O_2^- (superoxide) (Glupov et al., 2001) and its dismutation product, H_2O_2, are present in plasmatocytes of *Drosophila melanogaster* larvae (Nappi & Vass, 1998) and *G. mellonella* (Slepneva et al., 1999; Krishnan et al., 2008). The similarities between the oxidative burst pathways of insect haemocytes and mammalian neutrophils raise the possibility that the complexes that generate ROS might also contain homologous components. Recent reports provide evidence of the involvement of proteins homologous to human neutrophil p47phox and p67phox (Banfi et al., 2003; Geiszt et al., 2003). In this study Amplex Red reagent, an ultrasensitive method for detecting the production of H_2O_2, is used to provide evidence of H_2O_2 production by the haemocytes of *G. mellonella* larvae.

MATERIAL AND METHODS

Buffers and reagents

Three buffers were used in this study: Hank's balanced salt solution (HBSS, in mmol/l: NaCl 140, KCl 5.40, $NaHCO_3$ 4.17, $CaCl_2$ 1.26, $MgSO_4 \cdot 7H_2O$ 0.81, KH_2PO_4 0.44, $Na_2HPO_4 \cdot 12H_2O$ 0.36, glucose 5.05; pH 7.4), physiological solution for humans (PS) (0.9% solution of NaCl, pH 5.5) and insect saline (IS, in mmol/l: KCl 30, $NaHCO_3$ 5, $CaCl \cdot 2H_2O$ 8, $MgCl \cdot 6H_2O$ 15, KH_2PO_4 5 and sucrose 220, pH 6.6). Phenylthiourea (PTU) and Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) were purchased from Sigma (USA), luminol (dissolved in borate buffer, pH = 9.0) from Molecular Probes (USA) and L-012 (stock solution prepared in borate buffer) from Wako Pure Chemical Industry (Japan). Dextran-T500 (Pharmacia, Sweden) and telebrix N 300 (Léčiva, Czech Republic) were used for isolating leucocytes. Zymosan particles (ZP – Zymosan A from *Saccharomyces cerevisiae*; Sigma, USA) and zymosan particles opsonised by human serum (OZP), phorbol-12-myristate-13-acetate (PMA; Sigma, USA), N-formyl-methionyl-leucyl-phenylalanin (fMLP) or calcium ionophore (Ca-I A23187; Sigma, USA) were used as activators of ROS production. All

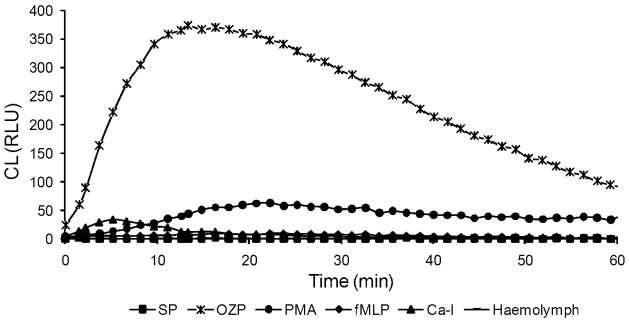

Fig. 1. The kinetics of the chemiluminescence (CL) reaction recorded during ROS production by human blood and haemolymph. Production by blood that occurred spontaneously (SP) or after stimulation with opsonised zymosan particles (OZP), phorbol-12-myristate-13-acetate (PMA), N-formyl-methionyl-leucyl-phenylalanin (fMLP) or calcium ionophore (Ca-I) was measured. Production by haemolymph was measured under the same conditions, but only one curve corresponding to all background level results is displayed.

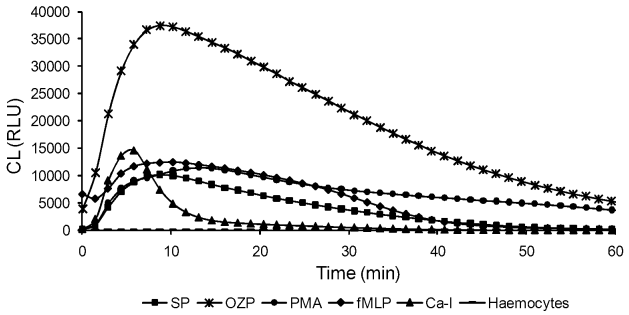

Fig. 2. The kinetics of the chemiluminescence (CL) reaction recorded during ROS production by isolated leucocytes and haemocytes. Production by leucocytes, resuspended in HBSS buffer with luminol that occurred spontaneously (SP) or after stimulation with opsonised zymosan particles (OZP), phorbol-12-myristate-13-acetate (PMA), N-formyl-methionyl-leucyl-phenylalanin (fMLP) or calcium ionophore (Ca-I) was measured. Production by haemocytes was measured under the same conditions, but only one curve corresponding to all background level results is displayed.

other chemicals were purchased from local distributors, and all the chemicals and reagents used were of reagent grade and the highest purity.

Isolation of haemocytes from insects

Galleria mellonella L. (Lepidoptera: Pyralidae) larvae (VIIth instar, 3–4 days old, 165.32 ± 2.4 mg) were obtained from laboratory cultures maintained on an artificial diet (Haydak, 1936) and kept at 28 ± 1°C in constant darkness. Haemolymph with haemocytes from larvae was collected by amputation of a proleg and pooled in a cooled tube containing several crystals of PTU. The haemocytes in haemolymph were diluted with buffers (HBSS, IS or PS) and the haemocytes were separated from haemolymph by centrifugation (500× g, 5 min at 4°C) prior to assay. Haemocyte counts were determined using a Bürker's chamber and adjusted (using HBSS, IS or PS) to a final concentration of 1.5×10^6 cells/sample (15-fold higher concentration than in human blood phagocytes).

Isolation of leucocytes from human blood

The heparinized blood (Na-heparin, 50 IU/ml) from healthy volunteers was obtained by antecubital venepuncture. Erythrocytes were removed after 1 h sedimentation in dextran/telebrix separation solution. For isolation of all the leucocytes, the leucocyte rich plasma (buffy coat) was centrifuged (300× g, 20°C, 5 min). The pellet was exposed to the hypotonic lysis of contaminating erythrocytes; the rest of the leucocytes were washed in HBSS and the total leucocyte counts adjusted to 1×10^6 cells/ml.

Chemiluminescence assay

The chemiluminescence (CL) of haemocytes and human whole blood were measured in duplicate using the microtitre plate luminometer LM-01T (Immunotech, Czech Republic) according to Pavelkova & Kubala (2004). Each reaction mixture contained 1×10^5 leucocytes or 1.5×10^6 haemocytes diluted in HBSS, IS or PS (appropriate solution), 1 mM luminol or 40 µM L-012 and 25 µl of one of the activators (OZP in a concentration of 62.5 µg/ml or ZP of 62.5 µg/ml for haemocytes, PMA in a concentration of 0.81 µg/ml, CaI in a concentration of 9.55 µg/ml or fMLP in a concentration of 1.14 µg/ml). Preparation of the stock solutions of luminol and oxidative burst activators follows Pavelkova & Kubala (2004). HBSS, PS or IS were used to adjust the total reaction volume to 250 µl.

Fluorescence assay

In the presence of peroxidase, the Amplex Red reagent reacts with H_2O_2 in a 1 : 1 stoichiometry to produce the red-fluorescent oxidation product, resorufin. Amplex Red solution (5 µl of 10 µM, stock solution in DMSO) was added to 10 ml of the appropriate solution with 10 units of horseradish peroxidase (HRP). The fluorescence (FL) of haemocytes or human leucocytes was measured using the microtitre plate fluorimeter Infinite 200 (Austria) at 585 nm. Firstly the number of haemocytes was optimized in a ratio 0.5–2 × 10^6 haemocytes per sample and for all subsequent experiments the concentration 1.5×10^6 haemocytes per sample was selected. Human leucocytes at a concentration of 1×10^5 per sample were used as a positive control. Each reaction mixture consisted of a total volume of 500 µl containing leucocytes or haemocytes (diluted in appropriate solution with Amplex Red) and 25 µl of one of the activators and was incubated for 20 min in the dark.

Statistical analysis

The results from the fluorescence assay are expressed as the mean ± standard error (SEM), n = 4. The level of significance was analysed by one-way analysis of variance (ANOVA), followed by a Tukey's test, values after activation within one buffer group were compared to spontaneous fluorescence.

RESULTS

Determination of ROS by chemiluminescence

In the first set of experiments the production of ROS by haemolymph or plasma (e.g. with or without haemocytes) was measured. Whole human blood was used as a positive control. No CL signal was detected in the haemolymph although several different diluents (Hank's balanced salt solution – HBSS, physiological solution – PS or insect saline – IS) and activators (zymosan particles – ZP, phorbol-12-myristate-13-acetate – PMA, N-formyl-methionyl-leucyl-phenylalanin – fMLP or calcium ionophore – Ca-I) in various concentrations were used. In contrast, human blood shows a typical kinetic reaction (Fig. 1) with opsonised zymosan particles (OZP) as the best activator.

Subsequently haemocytes were isolated to eliminate potential interference with other haemolymph components. Similarly, leucocytes from whole human blood were isolated as a positive control (Fig. 2). Also under these conditions, the CL signal for

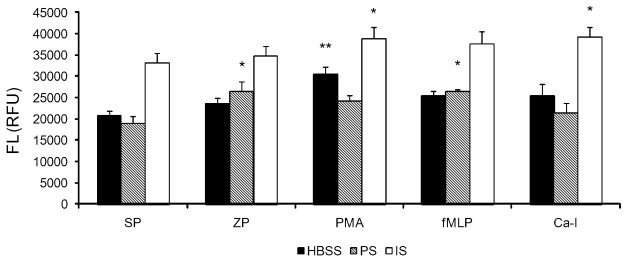

Fig. 3. Fluorescence (FL) determination of H_2O_2 production by haemocytes using different diluents (Hank's balanced salt solution – HBSS, physiological solution – PS, insect saline – IS) and activators: zymosan particles (ZP), phorbol-12-myristate-13-acetate (PMA), N-formyl-methionyl-leucyl-phenylalanin (fMLP) or calcium ionophore (Ca-I). Data are expressed as mean ± SEM, n = 5, significance level when compared with spontaneous FL (SP) * p = 0.05, ** p = 0.01.

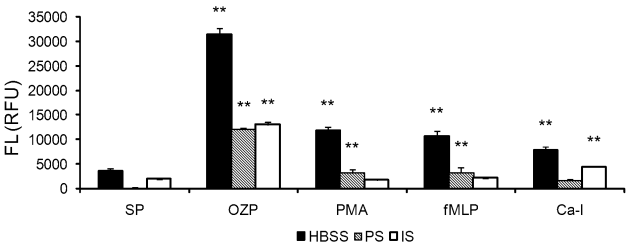

Fig. 4. Fluorescence (FL) determination of H_2O_2 production by human leucocytes using different diluents (Hank's balanced salt solution – HBSS, physiological solution – PS, insect saline – IS) and activators: opsonised zymosan particles (OZP), phorbol-12-myristate-13-acetate (PMA), N-formyl-methionyl-leucyl-phenylalanin (fMLP) or calcium ionophore (Ca-I). Data are expressed as mean ± SEM, n = 4, significance level when compared with spontaneous FL (SP) ** p = 0.01.

haemocytes remains at the background level in contrast to the ROS production by leucocytes, which show typical kinetics for individual activators (Fig. 2), and again OZP resulted in the greatest activation. To increase the sensitivity of the method, a stronger luminophor L-012 was also tested, however no CL signal was detected from haemocytes.

Determination of hydrogen peroxide by flourescence

Due to the negative results for ROS determination in haemocytes by CL, fluorescence was used as a more sensitive method in subsequent experiments.

The H_2O_2 production by *G. mellonella* haemocytes after incubation with Amplex red in different buffers and after activation by different activators is shown in Fig. 3. The best H_2O_2 production by spontaneous or activated haemocytes was detectable using IS buffer (pH 6.6), otherwise the FL signal was reduced significantly by an average of 32% with HBSS (pH 7.4, $p < 0.01$, only PMA $p < 0.05$) or 39% with PS (pH 7.4, without Ca^{2+} and Mg^{2+} ions, $p < 0.01$). Two activators show similar significant ($p < 0.05$) activation of haemocytes in IS (PMA 38908 ± 2570 RFU and Ca-I 39394 ± 2087 RFU) compared with spontaneous H_2O_2 production (33287 ± 2192 RFU, Fig. 4), while that of ZP (34819 ± 2278 RFU) and fMLP (37628 ± 2859 RFU) did not differ from spontaneous H_2O_2 level. Because the effect of activators on haemocytes was very small compared to human leucocytes, one half or 2–4× higher concentrations were also used to verify the optimal concentration, but H_2O_2 production decreased (low concentration of activator or higher toxicity at higher concentrations, data not shown).

The same measurement was repeated with human leucocytes (Fig. 4). In this case the highest H_2O_2 production was detected in spontaneous or activated leucocytes diluted in HBSS, the FL signal was significantly ($p < 0.01$) reduced by an average of 70% when PS without Ca^{2+} and Mg^{2+} ions and a different pH was used, similarly ($p < 0.01$) IS buffer (containing Ca^{2+} and Mg^{2+} ions, but lower pH than HBSS) caused a reduction in the FL signal by an average of 60%. All activators show significant ($p < 0.01$) activation of leucocytes in HBSS compared with spontaneous H_2O_2 production (3635 ± 417 RFU). Among the activators, the best stimulatory effect on human leucocytes was obtained using OZP (31482 ± 1179 RFU) followed by PMA (11904 ± 748 RFU), fMLP (10577 ± 1153 RFU) and Ca-I (7887 ± 552 RFU).

In summary, fluorescence and Amplex Red reagent was more sensitive for measuring insect haemocyte H_2O_2 production than luminometry. Elicitors resulted in different levels of haemocyte activation, but future optimization is needed to confirm these differences.

DISCUSSION

Previous studies have reported the production of the superoxide radical (Bergin et al., 2005; Slepneva et al., 1999) and nitric oxide (Krishnan et al., 2006) by *G. mellonella*. This study focused on the detection of ROS (H_2O_2 mainly) in *G. mellonella* haemocytes using common chemiluminescence and fluorescence.

Firstly chemiluminescence using luminol as luminophor was used for ROS detection in haemocytes with human blood as a positive control. Activators with different modes of action were applied to enhance the CL signal (Hoidal et al., 1978; Lad et al., 1985; Pohl et al., 1990). ZP or OZP particles are recognized by phagocyte receptors resulting in the activation of signal pathways including proteinkinase C (PKC), which catalyse endogenous protein phosphorylation followed by activation of NADPH-oxidase (the key enzyme of the respiratory burst). fMLP activator is a bacterial chemotactic polypeptide that activates signal pathways in connection with PKC and NADPH-oxidase by specific receptor binding. PMA passes through the cytoplasmatic membrane and directly activates PKC. Calcimycin A23187 (CaI) both increases intracellular calcium and activates PKC. However, no CL signal from haemolymph was detected when these two activators were used. In contrast, stimulated human blood phagocytes produced detectable levels of ROS. Haemocytes and blood phagocytes, respectively, were then isolated because components of haemolymph can interfere with the reaction (Dubovskii et al., 2010). A concentration of 1.5×10^6 per sample (15-fold higher amount of haemocytes than human leucocytes) was selected for subsequent measurements and human leuocytes were again used as a positive control. However, under these conditions the signal from isolated haemocytes remained at the background level in contrast to the ROS production by leucocytes. Additional approaches included using greater numbers of haemocytes, different buffers, more sensitive luminophor (L-012 instead of luminol – Daiber et al., 2004) and a higher concentration of activator. None of these changes led to detectable ROS production by haemocytes, which confirm previous luminometry results on *Bombyx mori* (Hyršl et al., 2004).

Fluorimetric ROS detection was used because it is a more sensitive method as it measures accumulated ROS rather than the level at one point in time, like CL. This method was used successfully e.g. for the measurement of the oxidative burst in lobster haemocytes (Moss & Allan, 2006). The fluorescent rea-

gent Amplex red was selected since it is a commonly used reagent for hydrogen peroxide (H_2O_2) production in mammalian cells, but there is only one report of it having been used for insects (Krishnan et al., 2008). Isolated haemocytes and human leucocytes showed a detectable fluorescence signal. After optimizing the number of haemocytes to a range of 0.5–2 × 10^6, a final concentration of 1.5 × 10^6 per sample was selected, because measured values of H_2O_2 production were comparable with that produced by leucocytes (but the number of leucocytes was 15-fold lower). Using these conditions different buffers and activators were tested. HBSS is the standard buffer designed for human blood cells with pH 7.4 and contains Ca^{2+} and Mg^{2+} ions, which are necessary for NADPH oxidase activation with subsequent ROS production (Brookes et al., 2004; Konrad et al., 2004; Touyz, 2004). Other buffers used include PS without Ca^{2+} and Mg^{2+} ions, with pH 5.5 and IS buffer designed for insect haemocytes, with pH 6.6, which is similar to the pH of haemolymph, 6.4–6.8 (Florkin & Jeuniaux, 1974; Mullins, 1985). Composition of IS is similar to HBSS as it also contains Ca^{2+} and Mg^{2+} ions.

Insect haemocytes produced the highest amount of H_2O_2 in IS buffer. In HBSS or PS the production of ROS was greatly decreased. These results indicate that haemocytes are not equally sensitive to pH changes and the presence of Ca^{2+} and Mg^{2+} ions is not as important as in human leucocytes. The stimulatory effects of different activators are not as pronounced as in human leucocytes, but statistical analysis confirmed significant differences in the activation of H_2O_2 production when using PMA and Ca-I (in the case of PMA also using HBSS buffer). Thus activator concentrations optimized for human cells (Pavelkova & Kubala 2004) are not suitable for insect haemocytes, thus different concentrations were tested. However, both lower and higher concentrations (one half and double, respectively) resulted in a lower H_2O_2 production. Higher concentrations of activators can be also be toxic for cells (Przygodzki et al., 2005) thus the concentration used in this study seems to be optimal for haemocytes.

For human leucocytes the highest production of H_2O_2 was observed using HBSS, which was expected as it is a special preparation for mammalian cells. Using IS and PS buffers with different compositions and pHs the H_2O_2 production led to a decrease in the signal. Thus, for human leucocytes it is more important that the pH is 7.4 and Ca^{2+} and Mg^{2+} ions are present. Regarding activators, the one with the best stimulatory effect on H_2O_2 production was OZP followed by PMA, fMLP and Ca-I.

To conclude, this study presents an ultrasensitive method, which uses Amplex Red reagent for determining H_2O_2 in insect haemocytes. The results show that the haemocytes of *G. mellonella* produced a low level of ROS. It is likely that ROS-production contributes less to direct bactericidal activity than to signalling pathways regulating immune reactions.

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