ORIGINAL ARTICLE

Effect of the extract from salivary glands of *Lucilia sericata* on human neutrophils

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Abstract

OBJECTIVES: There is incomplete information about host immune response to maggot therapy, nowadays increasingly used to clean chronic wounds from necrotic debris and infection. Maggots are applied to the wound during the inflammatory phase. At the same time neutrophils infiltrate the inflammatory site as the first defense line of the organism. Myeloperoxidase (MPO) and reactive oxygen species, generated during the respiratory burst by neutrophils, are the key players participating in microbial killing as well as in signalling pathways.
AIM: We studied the effect of an extract from salivary glands (SGE) of *Lucilia sericata* (*L. sericata*) on opsonized zymosan stimulated whole blood chemiluminescence (CL), superoxide generation and MPO release from human neutrophils.
METHODS: Formation of reactive oxygen species in whole blood was determined by luminol-enhanced CL. superoxide generation was measured as superoxide dismutase inhibitable reduction of cytochrome c, MPO activity as the oxidation of o-dianisidine in the presence of hydrogen peroxide.
RESULTS: Crude SG extract of *L. sericata* had no significant effect either on superoxide generation and MPO release from isolated unstimulated human neutrophils or on activity of isolated enzymes. Crude SG extract of *L. sericata* in the highest concentration used significantly decreased opsonized zymosan (0.5 mg/ml) stimulated blood CL, superoxide generation and MPO release.
CONCLUSION: On the basis of our results as well as from the literature we suggest that the beneficial effects of maggot therapy might involve the decrease of generation and release of proinflammatory factors, while neither phagocytosis nor subsequent apoptosis is disturbed.

Abbreviations

MPO - myeloperoxidase
SG - salivary glands
L. sericata - *Lucilia sericata*
CL - chemiluminescence
HRP - horseradish peroxidase
PBS - phosphate buffered saline

INTRODUCTION

Neutrophils are critical components of our innate immunity. They are a source of enzymes able to participate in reactive oxygen species generation. Reactive oxygen species and underlying regulatory factors play an important role in many physiological processes, including defense, immune and inflammatory responses (Maes *et al*., 2007; Valko *et al*., 2007) yet they may also deteriorate inflammation and wound healing (Dovi *et al*., 2004).

Alternative methods to handle chronic wounds, as reported by Vojtassak *et al*. (2007), are at least equivalent if not better than conventional therapy. Concerning chronic wounds infected with bacteria and other microorganisms multiresistant to antibiotics, there is renewed interest for maggot therapy (Sherman *et al*., 2000; Cambal *et al*., 2006). Medicinal maggots of the green bottle fly *L. sericata* work as biochemical debriding agents: they operate with precision at the boundary between healthy and necrotic tissue. Through a process known as "extracorporeal digestion", they secrete a broad spectrum of proteolytic enzymes liquefying necrotic tissue, and other biologically active compounds disinfecting the wound and stimulating wound healing (Nigam *et al*., 2006). They also ingest bacteria and degrade them in their intestinal tract. Maggots have a repertoire of externalized defenses against microbes, most of which have yet to be identified. Isolation, identification, characterisation and synthesis of maggot-derived antibacterial compounds may open new therapeutic possibilities.

We investigated the effects of crude SG extract of *L. sericata* on whole blood CL, MPO release and superoxide production, functional responses of human neutrophils participating in antimicrobial activities.

MATERIAL AND METHODS

Luminol (Sigma-Aldrich), Cytochrome c, horseradish peroxidase (Merck), Dextran T500 (Pharmacia Fine Chemicals). Lymphoprep (Nycomed Pharma AS), opsonized zymosan (Sigma). All other chemicals were purchased from Sigma-Aldrich.

Isolation of human neutrophils

Blood was collected by venipuncture from healthy male volunteers who had abstained from any drugs for at least one week before sampling. Blood was taken into 3.8% trisodium citrate, erythrocytes were removed by dextran sedimentation and centrifugation on Lymphoprep by the modified Boyum's method (Jančinová, 2006). Neutrophils for superoxide and MPO determination were 1×10^6 and 2×10^6 /sample, respectively.

Salivary gland sample preparation and purification

Larvae of the third larval instar of *L. sericata* were immobilised on ice and SG were dissected under a stereomicroscope. Salivary glands, 20 pairs, were placed in 140 µl ice-cold PBS (0.01 mol/l phosphate buffer and 0.15 mol/l NaCl, pH 7.2), heated to 80 °C for 5 min, then homogenised and centrifuged at 10 000 g for 10 min. The supernatant in concentration of 1 salivary gland per 1 µl of frozen supernatant, termed SG extract [1 SG/1 µl], was stored at –70 °C before use in the experiments.

Whole blood chemiluminescence

CL of blood was measured, using luminol-enhanced CL, in a microtitre plate computer driven luminometer LM-01T at 37 °C according to Drábiková *et al*. (2006). Measurement of CL was started by addition of opsonized zymosan (0.5 mg/ml) to the reaction mixture, which consisted of luminol (final concentration 250 µmol/l), whole blood (1 µl/sample), PBS, SG extract) in final volume 250 µl. CL was recorded continuously for 30 min to obtain kinetic curves and evaluated on the basis of integral values (RLU*s; RLU – relative light units).

Zymosan

Zymosan A from Saccharomyces cerevisiae was opsonized according to Lojek *et al*. (2002) in pooled serum, washed three times and frozen at –28 °C. Immediately before use it was thawed, diluted and added to the cell suspension to have a final concentration of 0.5 mg/ml.

Superoxide determination

Superoxide formation was measured in isolated human neutrophils as superoxide dismutase inhibitable reduction of cytochrome c. Neutrophils were preincubated 5 min at 37 °C with SG extract (final concentration 0.0835; 0.167 SG/sample) and subsequently stimulated by addition of 0.5 mg/ml opsonized zymosan for 60 min at 37 °C. Controls were included for the effect of stimulus and extract from SG on cytochrome c reduction. Absorbance was measured at 550 nm in a microplate spectrophotometer. The superoxide production was calculated as described (Pečivová *et al*., 2007).

Myeloperoxidase release

Neutrophils were preincubated with the extract in a shaker bath at 37 °C for 5 min, followed by 60 min exposure to opsonized zymosan. The activity of MPO was assayed in the supernatant after centrifugation 2 000×g for 10 min at 4 °C by determining the oxidation of o-dianisidine in the presence of hydrogen peroxide according to Pečivová *et al*. (2006).

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). The data were analysed by Student's t-test and $p \leq 0.05$ was taken as significant.

RESULTS

In preliminary experiments we tested the concentration dependence of the extract from SG (data not shown). SG extract had no significant effect on whole blood CL, superoxide generation and MPO release from unstimulated human isolated neutrophils. Concentrations of SG extract lower than 0.0835 SG/sample had no effect on the parameters measured after stimulation. Figure 1 shows the resulting significant changes of whole blood CL, superoxide and MPO release stimulated by opsonized zymosan. For comparison, the effect is given in percentages. The concentration of SG extract [0.0835 SG/sample] decreased, though n.s., the factors measured: whole blood CL, superoxide generation and MPO release by 5.63%, 3.66%, 5.76%, respectively. To differentiate the membrane effect of the extract from its direct effect on MPO activity, we compared its effect on cell free system: crude extract from neutrophils and horse radish peroxidase. The extract had no significant effect on the activity of the isolated enzyme (Figure 2).

DISCUSSION

Maggots of some Dipteran species are used to disinfect chronic wounds by mechanically removing bacteria and by releasing compounds that kill microorganisms (Bexfield *et al*., 2004; Chan *et al*., 2007). The aim of our study was to analyse effects of extracts from salivary glands of *L. sericata* on whole human blood CL and two functional responses of isolated human neutrophils: superoxide generation and myeloperoxidase release. Opsonized zymosan, a membrane receptor operating stimulus (different regulatory pathway compared to fMLP and PMA), evoked respiratory burst, measured as blood CL, superoxide generation, and MPO release as a marker of degranulation. SG extract dose dependently decreased all responses stimulated by opsonized zymosan, yet significantly only in the highest concentration used. The highest SG extract effect measured was on whole blood CL. This may be the effect of inhibition of superoxide generation together with decrease of reactive oxygen species generation connected with MPO release, but before the identity of bioactive molecules conteined in SG extract will be known, it can not be excluded that some compound of SG extract has an intrinsic antioxidant/scavenging activity as well. Dose dependence of the effects of maggot excretions/secretions measured as H_2O_2 production, intracellular cAMP concentration were reported for another physiological stimulus, i.e. fMLP (receptor) and a non-physiological stimulus – phorbol myristate acetate activating proteinkinase C (Van der Plas *et al*., 2007). The ability to influence β_2 integrins CD11b and CD18 and neutrophil migration towards fMLP was also observed. Our results are in agreement with the above findings. We moreover established a decrease of opsonized zymosan stimulated reactive oxygen species generation, supported by decrease of MPO release, which is another potential source of reactive oxygen species generation and also a powerful autocrine and paracrine stimulator of neutrophil and similarly activator of other inflammatory cell closely linked to the progression of chronic inflammatory diseases (Lau, 2005). It may be concluded that extracellular release of inflammatory mediators is

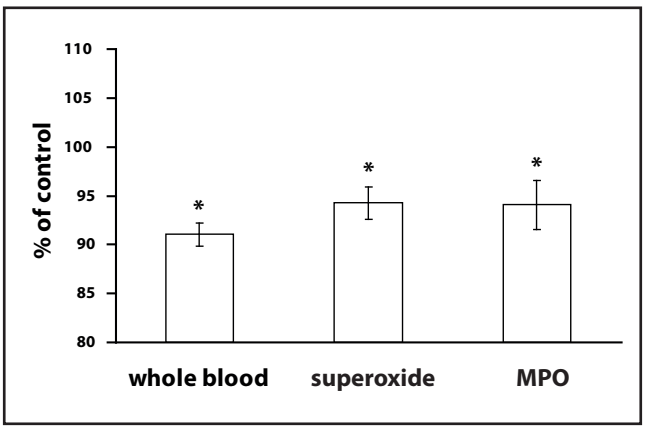

Figure1. Effect of SG extract [0.167 SG/sample] on opsonized zymosan [0.5 mg/ml] stimulated whole blood CL, superoxide generation and myeloperoxidase release versus control without the drug. Results are mean SEM, n = 4–6, *p<0.05. For opsonized zymosan, the absolute average control value of whole blood CL, superoxide generation and myeloperoxidase release was 26 915 ± 2 168 RLU*s, 13.1 ± 2.14 nmoles/10^6 neutrophils/min and 4.11 ± 0.31 ΔA/Δt (calculated as area under curve), respectively.

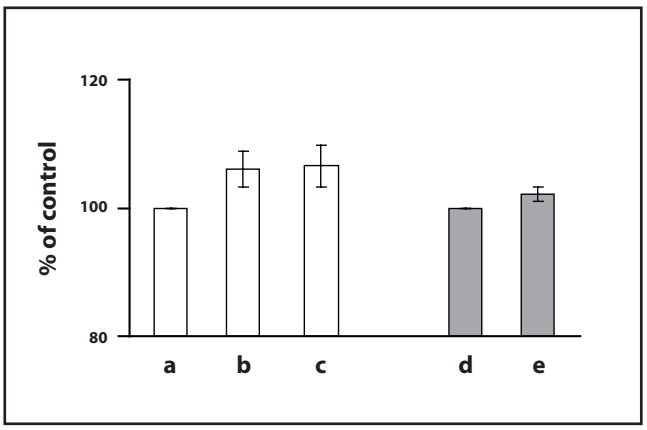

Figure 2. Effect of SG extract [0.167 SG/sample] and opsonized zymosan [0.5 mg/ml] on peroxidase activity versus non treated control (**a** = MPO [2×10^6 neutrophils]; **b** = MPO + SG extract; **c** = MPO + SG extract + opsonized zymosan; **d** = HRP [2 U/sample]; **e** = HRP + SG extract). Results are mean ± SEM, n=3.

decreased but the ability to use the microbicidial armamentory intracellularly is retained. Thus the effects of neutrophils and maggots are not contradictory but rather supportive in cleaning and heeling chronic wounds.

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