Maggot therapy´s modes of action : effect of maggot secretions on microbiological, haematological and immunological processes

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CHAPTER FIVE

Maggot excretions/secretions inhibit multiple neutrophil pro-inflammatory responses

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Abstract

There is renewed interest in the use of maggots (*Lucilia sericata*) to aid in healing of chronic wounds. In such wounds neutrophils precipitate tissue damage rather than contribute to healing. As the molecules responsible for the beneficial actions of maggots are contained in their excretions/secretions (ES), we assessed the effects of ES on functional activities of human neutrophils.

ES dose-dependently inhibited elastase release and H_2O_2 production by fMLP-activated neutrophils; maximal inhibition was seen with 5-50 µg of ES/mL. In contrast, ES did not affect phagocytosis and intracellular killing of *Candida albicans* by neutrophils. Furthermore, 0.5 µg of ES/mL already inhibited neutrophil migration towards fMLP. ES dose-dependently reduced the fMLP-stimulated expression of CD11b/CD18 by neutrophils, suggesting that ES modulate neutrophil adhesion to endothelial cells. ES did not affect the fMLP-induced rise in $[Ca^{++}]_i$ in neutrophils, indicating that ES act down-stream of phospholipase C-mediated activation of protein kinase C. In agreement, ES inhibited PMA-activated neutrophil functional activities. ES induced a rise in intracellular cAMP concentration in neutrophils and pharmacological activators of cAMP-dependent mechanisms mimicked their inhibitory effects on neutrophils.

The beneficial effects of maggots on chronic wounds may be explained in part by inhibition of multiple pro-inflammatory responses of activated neutrophils by ES.

Introduction

For hundreds of years the beneficial effects of maggots on wound healing have been documented[1]. The systemic use of maggots in patients started in the late 1920s by William Baer, who successfully applied them to patients with osteomyelitis[2] and rapidly thereafter the use of maggots for treating wounds became widespread. However, by the mid-1940s maggot therapy was abandoned due to the introduction of antibiotics and improved surgical techniques. In the 1980s, maggot therapy made its comeback with the emergence of antibiotic-resistant bacteria and nowadays larvae of the green bottle blowfly *Lucilia sericata* are used worldwide for the treatment of many types of wounds, including venous ulcers[3], traumatic and post-surgical wounds[4], osteomyelitis[5] and burns[6]. The molecules involved in the beneficial effects of maggots are believed to be contained in their excretions/secretions (ES). Clinical observations indicate that in addition to removal of necrotic tissue, maggots promote wound healing, especially in wounds that show little tendency to heal. Wound healing is a complex well-orchestrated repair process that comprises three phases: inflammation, proliferation and remodelling. Although maggots are applied during the inflammatory phase, there is little information available about the effects of maggots on the cells that characterize the wound in this phase.

Neutrophils are an essential component of the inflammatory response in wounds. These cells are recruited from the circulation to the affected site where they are essential to combat infections[7]. Most inflammatory responses resolve after clearance of bacteria from tissues, but in chronic wounds there appears to be a continued presence of inflammatory leucocytes, most notably neutrophils[8,9], that may accelerate tissue damage by excess production and release of bioactive substances like proteinases and reactive oxygen species. Based on the above considerations the aim of this study was to investigate the effects of ES on multiple human neutrophil pro-inflammatory activities.

Materials and methods

Preparation of maggot excretions/secretions

Sterile second- and third-instar larvae of *L. sericata* were a kind gift from BioMonde GmbH (Barsbüttel, Germany). Maggot ES were collected after incubating approximately 50 larvae/tube in 200 µL of Milli-Q ultrapure water for 60 min at ambient temperature in the dark. Next, ES was checked for sterility and stored at -20°C. Prior to use, ES preparations were pooled in 15 mL tubes (Greiner Bio-One, Alphen aan de Rijn, The Netherlands) and centrifuged at 1,300xg for 5 min at 4°C to remove particulate material. ES protein concentration was determined using the Pierce BCA Protein Assay kit according to manufacturer's instructions.

Isolation of human neutrophils

Neutrophils from healthy donors were isolated from fresh venous blood transferred to 50 mL tubes (Greiner Bio-One) by Ficoll Amidotrizoate (ρ = 1.077 g/mL) density centrifugation at 440xg for 20 min. Erythrocytes were removed from the cell pellets by hypotonic lyses using a buffer (pH 7.2) containing 0.1 mM EDTA, 0.18 M NH_4Cl and 10 mM $KHCO_3$. The final cell suspensions consisted of 97 ± 2% neutrophils and the cell viability amounted to 95%, as determined by trypan blue exclusion.

Stimulation of neutrophils

Neutrophils were stimulated with the indicated concentrations of formyl-Met-Leu-Phe (fMLP; Sigma Chemical Co., St. Louis, MO, USA) or 100 ng/mL phorbol-12-myristate-13-acetate (PMA; Sigma). To establish the effects of ES, neutrophils were incubated with these stimuli together with increasing concentrations of ES (range 0.5-100 µg/mL). Furthermore, ES was boiled for 2 h to investigate the heat-stability of the active molecules.

Measurement of elastase release

Neutrophils were resuspended in 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, 1.5 mM KH_2PO_4, and 2.5 mg of BSA/mL to a concentration of $5x10^6$ cells/mL and then incubated with 100 nM fMLP or PMA and ES or no stimulus at 37°C for 30 min. The reaction was stopped by transferring the cells onto ice. After centrifugation at 100xg for 5 min, the cell-free supernatants were transferred to equal volumes of a buffer containing 70 mM Tris, 700 mM NaCl and 0.2% (wt/v) gelatin and the absorbance was read at 405 nm. Subsequently, 1/10 volume of substrate (Pefafluor®ELA-5534; Penthapharm Ltd, Basel, Swiss) was added and the absorbance by the reaction product was read every 15 min up to 60 min. Results are expressed as arbitrary units (a.u.)/$2.5x10^5$ cells after 30 min incubation. Values were corrected for the enzymatic activity present in ES[10], which was determined in cell-free experiments. To enhance responsiveness to fMLP, the neutrophils were preincubated with 10 µM cytochalasin B (Sigma) for 10 min[11].

Measurement of extracellular hydrogen peroxide

Extracellular release of hydrogen peroxide was measured as described[12]. Neutrophils ($1x10^7$ cells/mL) were resuspended in Hank's Buffered Saline Solution (HBSS) and preincubated with cytochalasin. Subsequently, $5x10^5$ cells were transferred to tubes containing HBSS supplemented with 100 µM homovanillic acid (Fluka, Buchs, Switzerland), 1 U horse reddish peroxidase (Sigma)/mL and 100 nM fMLP or PMA in combination with ES or as a control no stimulus. After 30 min incubation at 37°C in the dark, the reaction was stopped by adding 1/8 volume of glycine-NaOH buffer containing 46 mM glycine, 46 mM NaCl, 540 mM NaOH and 25mM EDTA. Next, tubes were centrifuged at 1,000xg for 10 min and the H_2O_2 production was determined by measuring the fluorescence of the supernatants

on a F4500 fluorescence spectrophotometer (Hitachi Europe GmbH, Germany) using an excitation wavelength of 312 nm and an emission-detection wavelength of 420 nm. The production of H_2O_2 by the cells was calculated using a standard curve constructed with various concentrations of H_2O_2. The results are expressed as nmol H_2O_2/5x10^5 neutrophils. Values were corrected for the amount of H_2O_2 in cell-free samples.

Chemotaxis assay

Migration of neutrophils in response to various stimuli was measured using a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, MD, USA) as described[13] with minor modifications. fMLP and ES or incubation buffer in a 1:1 ratio with RPMI 1640 (supplemented with 2 mM glutamax-I/glutamine and penicillin/streptomycin; RPMI-p/s/g) were added to the lower compartments. The incubation buffer consisted of 20 mM Hepes buffer (pH 7.4) supplemented with 0.5% HSA, 5.5 mM glucose and 1 mM $CaCl_2$. Next, two filters presoaked in incubation buffer were placed between the lower and upper compartments. The lower filter had a pore size of 0.45 µm (Millipore, Bedford, MA, USA) and the upper filter of 8 µm (thickness, 150 µm; Sartorius, Gottingen, Germany). Thereafter, neutrophils (25 µL of 2x10^6 cells/mL) were placed in the upper compartment. After incubation for 1.5 h at 37°C, the upper filters were removed, fixed in butanol/ethanol (20/80%) for 10 min, and stained with Weigert's solution. Subsequently, the filters were dehydrated with ethanol, made transparent with xylene, and fixed upside down onto microscope slides. For counting of the number of migrated cells, pictures were made of each level within the filters with a CoolSNAP camera (RS Photometrics, Roper Scientific BV, Vianen, the Netherlands) connected to an Olympus BX51 microscope (Olympus Nederland BV, Zoeterwoude, The Netherlands). The first level in the filter that contained neutrophils attracted by 10 nM fMLP was taken as the first level for all samples. Neutrophils were counted in 6 subsequent levels within each filter. Results are expressed as the average number of cells in a 2 μm^2 area/high power field.

Measurement of CD11b/CD18 expression

The effect of ES on the fMLP-induced expression of CD11b and CD18 by neutrophils was measured by FACS analysis. Neutrophils (2x10^6/mL) were suspended in RPMI-p/s/g containing 10% heat-inactivated foetal calf serum. Subsequently, 1 mL of this suspension was transferred to wells of a Costar 24-well cell culture plate and incubated with or without fMLP (1 µM) and ES or no stimulus at 37°C for 1 h. Neutrophils were harvested and washed with ice-cold PBS containing 0.2% (wt/v) BSA. Next, cells were labelled for 30 min on ice with monoclonal antibodies against CD11b ($mIgG_{1\kappa}$; DAKO A/S, Denmark) or CD18 (IB4, $mIgG_{2a}$; American Type Culture Collection, Manassas, VA, USA) in PBS/BSA, washed, and then incubated for 30 min with R-phycoerythrin-labelled goat anti-mouse Ig(H+L) (Southern Biotechnology Associates, Inc, Birmingham, Al, USA). Thereafter, cells were washed and

then analyzed on a FACSCalibur (Becton & Dickinson, La Jolla, Ca, USA). Results are median fluorescence intensities (MFI) of ES-incubated, fMLP-activated neutrophils relative to the MFI for fMLP-activated cells, as calculated using CellQuest^{tm} Pro 4.0.2 software (Becton & Dickinson).

Phagocytosis and killing assay

Phagocytosis and intracellular killing of *Candida albicans* Y01-19 (Pfizer, Groton, Conn, USA) by neutrophils was measured as described[14]. *C. albicans* were cultured for 5 days in Sabouraud broth at 30°C, then washed with PBS and resuspended in HBSS-0.1% gelatin. Equal volumes of this *C. albicans* suspension ($1.1x10^7$/mL) and a neutrophil suspension ($1.1x10^7$/mL HBSS-0.1% gelatin) were mixed. Subsequently, 85 µL of this mixture were transferred to NUNCLON™ Surface plates (Nalge Nunc International, Rochester, NY, USA) containing either 15 µL of HBSS-0.1% gelatin or serum derived from AB positive donors in combination with ES or no ES. At various intervals after incubation at 37°C under slow rotation, the number of non-cell-associated *C. albicans* was assessed using a Bürker hemocytometer. Phagocytosis is expressed as the percentage decrease of non-cell-associated *C. albicans*.

For assessment of intracellular killing, samples from the mixture were taken at various intervals and transferred to Eppendorf tubes containing H_2O supplemented with 0.01% (wt/v) BSA and 0.01% tween-20. Thereafter, these suspensions were vortexed for 30 s and then sonicated for one min. Next, the number of viable *C. albicans* in these samples was determined microbiologically. Killing is expressed as the percentage decrease in the number viable *C. albicans*.

Measurement of the $[Ca^{++}]_i$

For measurement of the $[Ca^{++}]_i$, $2x10^7$ neutrophils/mL of Ca^{++}-buffer (pH 7.4; 20 mM Hepes supplemented with 138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.1 mM $CaCl_2$, 1 mM NaH_2PO_4, 5.5 mM D-glucose, 0.1 mM EGTA, and 0.1% wt/v BSA) were incubated with 2 µM acetoxymethyl ester of FURA-2 (Sigma) for 30 min at 37°C in the dark[15]. Subsequently, cells were washed and resuspended in Ca^{++}-buffer. Changes in $[Ca^{++}]_i$ after exposure to ES or Ca^{++}-buffer and 5 min thereafter fMLP were measured using the F4500 spectrophotometer. At the end of each measurement the 340nm/380nm signals were calibrated using 0.2% Triton X-100 and 20 mM EGTA to determine the maximum and minimum free Ca^{++} concentration, respectively. Thereafter, the $[Ca^{++}]_i$ was calculated using the equation of Grynkiewicz[16].

Measurement of intracellular cAMP concentration

Neutrophils ($2x10^6$/20 µL) in HBSS were incubated with ES or fMLP (100 nM) or no stimulus for various intervals up to 2 min. The reaction was stopped by adding 10 volumes of lysis

buffer. Next, the cAMP content of these samples was measured using the cAMP Biotrak Enzymeimmunoassay System (Amersham Biosciences, Buckinghamshire, UK) according to manufacturer's instructions. The intracellular concentrations were calculated from these cAMP values and the mean cell volume of human neutrophils[17].

Cell viability

To check whether ES affect cell viability, neutrophils were incubated for 1 h and 24 h with ES, fMLP or no stimulus and then stained with FITC-labeled Annexin V (2.5 µg/mL, Sigma) and propidium iodide (1 µg/mL, Sigma) in 10 mM Hepes (pH 7.4) as described[18]. Thereafter, the fluorescence intensities of the cells were analyzed by flow cytometry.

Statistical analysis

Statistical analyses were performed using SPSS for Windows version 11.0. Differences between the values for cells stimulated with fMLP or PMA together with ES and those for neutrophils exposed to these stimuli alone were analysed with a one-way ANOVA and a Dunnett's posttest for multiple comparisons. The same approach was taken for analysis of the differences between cAMP-values for cells exposed to ES and those for non-exposed cells. Student's t-tests were used for analysis of the differences between values for cells exposed to fMLP and PMA together with db-cAMP and those for cells stimulated by fMLP or PMA alone. $P<0.05$ was considered significant.

Results

Effect of ES on elastase release and hydrogen peroxide production

Since tissue damage associated with chronic inflammation involves the action of proteases, like elastase, and reactive oxygen intermediates released by activated neutrophils, we first investigated the effects of ES on the release of these factors by fMLP- and PMA-activated neutrophils. The results revealed that ES dose-dependently inhibited elastase release by neutrophils in response to fMLP with maximal inhibition seen with 50 µg of ES/mL (Figure 1A). Elastase release by neutrophils upon PMA activation was not significantly affected by ES (Figure 1B). Control experiments revealed that ES did not trigger the elastase release by resting neutrophils; the proteolytic activity of ES amounted to $4x10^{-3}$ arbitrary units/µg.

Already 5 µg of ES/mL blocked the H_2O_2 production by neutrophils in response to fMLP (Figure 2A). ES inhibited the H_2O_2 production by PMA-activated cells in a dose-dependent fashion with maximal inhibition seen with 100 µg of ES (Figure 2B). Control experiments revealed that ES neither affected the H_2O_2 production by resting neutrophils nor interfered with the measurement of H_2O_2 when added to standard curves, indicating that it did not quench the fluorescence of homovanillic acid.

Interestingly, boiling ES completely abrogated its inhibitory effects on neutrophil degranulation and H_2O_2 production (data not shown), indicating that the active component(s) in ES is (are) heat-labile. Moreover, ES did not affect cell viability of neutrophils even at the highest dose used, i.e. 100 µg/mL (data not shown).

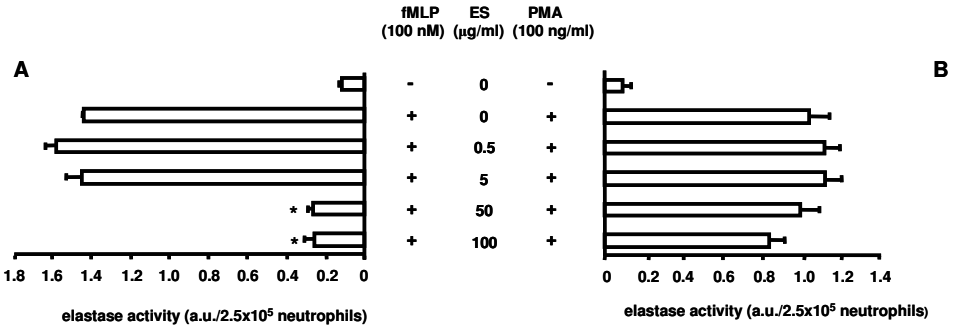

Figure 1 Effect of maggot excretions/secretions on elastase release by neutrophils in response to fMLP (A) and PMA (B). Briefly, neutrophils were incubated with fMLP (100 nM) or PMA (100 ng/mL) together with ES (range 0.5-100 µg/mL) or no stimulus. After incubation for 30 min at 37°C, the reaction was terminated and then the cells were centrifuged. The elastase activity in the medium was assessed using the chromogenic substrate Pefafluor®ELA-5543. At various intervals thereafter the amount of reaction product in the supernate was quantitated by measuring the absorbance at 405 nm. Results, expressed as arbitrary units (a.u.)/2.5x10^5 cells, are means ± SEM of six experiments. To enhance responsiveness towards fMLP, the cells were preincubated with 10 µM cytochalasin B. *Values are significantly ($p<0.05$) different from those for neutrophils stimulated with fMLP or PMA alone.

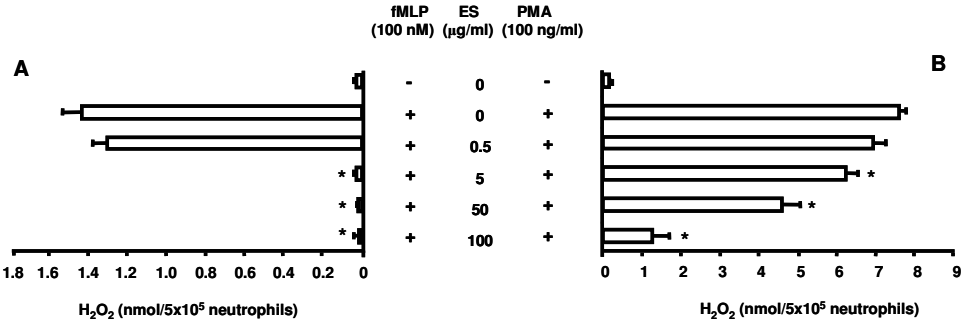

Figure 2 Effect of maggot excretions/secretions on the H_2O_2 production by neutrophils in response to fMLP (A) and PMA (B). The production of H_2O_2 by neutrophils upon stimulation with fMLP (100 nM) or PMA (100 ng/mL) in the presence of ES (range 0.5-100 µg/mL) or no stimulus was determined using the fluorescent probe homovanillic acid. After incubation for 30 min at 37°C, the reaction was stopped and the cells were centrifuged. Thereafter, the amount of the reaction product in the supernate was quantitated by measuring the fluorescence (excitation wavelength of 312 nm and emission of 420 nm). Results, expressed as nmol H_2O_2/5x10^5 cells, are means ± SEM of four to six experiments. To enhance responsiveness towards fMLP, the cells were preincubated with 10 µM cytochalasin B. *Values are significantly ($p<0.05$) different from those for neutrophils stimulated with fMLP or PMA alone.

Effect of ES on neutrophil migration towards fMLP

To investigate whether ES inhibited the neutrophil chemotaxis, we determined the effect of ES on the number of neutrophils migrating towards fMLP. The results revealed that ES dose-dependently inhibited the fMLP-stimulated neutrophil migration (Figure 3). A significant inhibitory effect was already observed with 0.5 µg of ES/mL while 100 µg/mL blocked the fMLP-induced neutrophil migration. Control experiments showed that ES was not chemotactic for neutrophils.

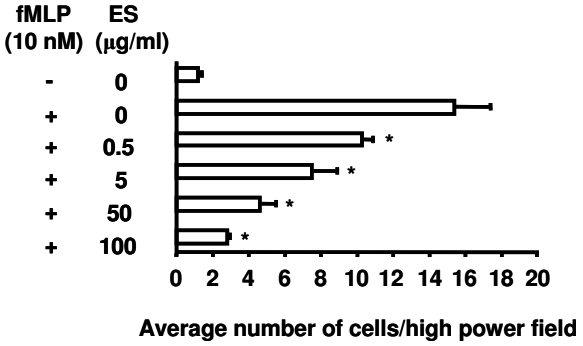

Figure 3 Effect of maggot excretions/secretions on the fMLP-stimulated neutrophil chemotaxis. Migration of neutrophils in response to 10 nM fMLP was monitored using a Boyden microchemotaxis chamber. In short, fMLP together with ES (range 0.5-100 µg/mL) or as a control with buffer was pipetted in the lower compartment and then the cells were applied to the upper compartment. After allowing the cells to migrate through the filters between the two compartments towards the lower compartment for 1.5 h at 37°C, the filters were removed and the number of cells in six subsequent high power fields was determined microscopically. Results, expressed as the average number of cells per high power field, are means ± SEM of three experiments. Each experiment was performed in quadruplicate. *Values are significantly ($p<0.05$) different from those for neutrophils stimulated with fMLP alone.

Effect of ES on the fMLP-induced expression of CD11b and CD18

Neutrophils must adhere to endothelial cells in order to migrate through the blood vessel wall into a wound. In this connection, we determined the effect of ES on the fMLP-stimulated expression of CD11b and CD18, molecules involved in the adherence of neutrophils to e.g. endothelial cells. The results revealed that ES dose-dependently inhibited the expression of CD11b and CD18 on fMLP-activated neutrophils, with 100 µg/mL of ES inhibiting respectively 67% and 90% (Figure 4). Furthermore, ES did not affect the expression of CD11b and CD18 on resting cells, indicating that ES do not cleave off these molecules of the cell surface.

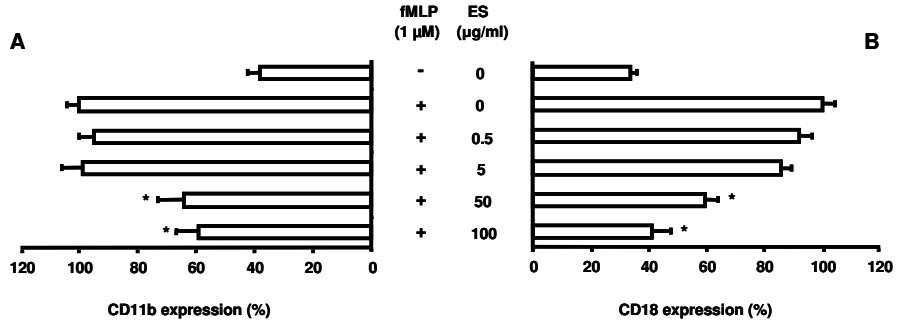

Figure 4 Effect of maggot excretions/secretions on fMLP-induced CD11b (A) and CD18 (B) expression by neutrophils. In short, neutrophils were incubated with fMLP (1 µM) together with ES (range 0.5-100 µg/mL) or as a control no ES for 1 hr at 37°C. Thereafter, cells were washed and reincubated for 30 min with fluorescently labelled monoclonal antibodies directed against CD11b or CD18, and as control no antibody, and finally the fluorescence intensity of the cells was quantitated by flow cytometry. Results, expressed as the median fluorescence intensity (MFI) of ES-incubated, fMLP-stimulated neutrophils relative to the values of fMLP-stimulated cells, are means ± SEM of four to six experiments. *Values are significantly ($p<0.05$) different from those for neutrophils stimulated with fMLP alone.

Effect of ES on the phagocytosis and killing of C. albicans

To investigate whether ES decreased the antimicrobial activities of neutrophils we determined its effects on phagocytosis and killing of *C. albicans* by these cells. The results showed that ES had no effect on the phagocytosis and killing of *C. albicans* by neutrophils (Table 1). Furthermore, ES did not influence these activities by neutrophils incubated without serum (data not shown). In addition, ES had no effect on the viability of *C. albicans* under these conditions.

Table 1 Effect of ES on phagocytosis and intracellular killing of *Candida albicans* by neutrophils.

ES (µg/mL)	Phagocytosis (%) at various intervals (min)			killing (%) at various intervals (min)		
	5	15	30	5	15	30
0	20 ± 5	62 ± 7	83 ± 2	31 ± 6	36 ± 8	45 ± 5
5	14 ± 6	55 ± 7	80 ± 2	29 ± 6	43 ± 7	44 ± 5
50	24 ± 9	58 ± 6	83 ± 3	36 ± 5	43 ± 8	48 ± 4

Equal numbers of neutrophils and *C. albicans* were incubated with 15% (v/v) serum derived from AB positive donors in the presence or absence of ES (5 and 50 µg/mL). At various intervals, the number of non-cell-associated *C. albicans* was determined using a Bürker hemocytometer. Phagocytosis is expressed as the percentage decrease in the number of non-cell-associated *C. albicans* and is mean ± SEM of four experiments. For assessment of killing, the number of viable *C. albicans* was measured by plating serial dilutions of the suspension. Killing is expressed as the percentage decrease in viable *C. albicans* and is mean ± SEM of four experiments. No significant differences were observed between the values for phagocytosis and killing of *C. albicans* by neutrophils incubated with ES and those for neutrophils not exposed to ES.

Effect of ES on the fMLP-stimulated rise in the $[Ca^{++}]_i$

Activation of neutrophils by fMLP involves its binding to a G-protein coupled receptor and subsequent activation of down-stream pathways[19]. A major pathway involves phospholipase C, which acts on phosphoinositol(4,5)biphosphate to produce inositol(1,4,5)trisphosphate, an activator of the release of Ca^{++} from specific intracellular stores into the cytosol, and diacylglycerol; together these two intracellular messengers activate protein kinase C. As ES inhibited fMLP-activated neutrophils we questioned whether ES interfered with the fMLP-induced rise in $[Ca^{++}]$ in neutrophils. Basal $[Ca^{++}]_i$ in neutrophils amounted to 94 ± 2 nM and the peak value seen after stimulation with fMLP to 572 ± 15 nM. ES did not stimulate a rise in the $[Ca^{++}]_i$ nor did it affect the kinetics of the fMLP-induced rise in $[Ca^{++}]_i$ in neutrophils (Figure 5).

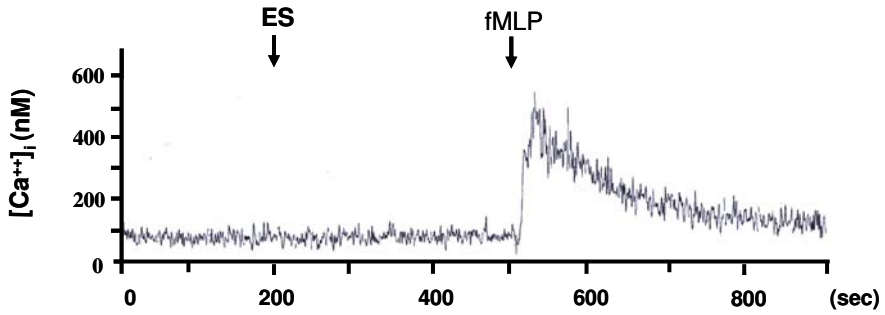

Figure 5 Effect of maggot excretions/secretions on fMLP-induced increase in the $[Ca^{++}]_i$ in neutrophils. Fura-2-loaded neutrophils were transferred to a quartz cuvette and then placed in a spectrophotometer. Thereafter, the samples were excited at 340 nm and 380 nm using an emission wavelength of 500 nm and the 340nm/380 nm ratio's were used to calculate the changes in $[Ca^{++}]_i$ in the cells in response to first ES or Ca^{++}-buffer as a control and 5 min thereafter to fMLP. Results are of a representative experiment out of three experiments.

Involvement of cAMP-dependent mechanisms in the inhibitory effects of ES

To investigate if cAMP-dependent mechanisms are involved in the inhibitory effects of ES on neutrophil responses, two sets of experiments were performed. First, we determined whether ES stimulate a rise in the intracellular cAMP concentration in neutrophils. The results revealed that 50 µg of ES/mL enhanced the intracellular cAMP concentration in neutrophils from 0.84 ± 0.10 µM to 1.26 ± 0.15 µM; the peak value after 100 nM fMLP amounted to 1.17 ± 0.03 µM (n=3-4). At 15 sec after addition of ES the peak intracellular cAMP concentration was reached and thereafter the values returned gradually to basal values at 60 sec (results not shown). Based on these data, the 15 sec interval was chosen to determine the dose-effect relation for ES. The results revealed that 5 and 50 µg of ES/mL both increased the intracellular cAMP concentration 1.5-fold over basal values and the

maximum increase (1.7-fold over basal values) was seen with 100 µg of ES/mL (Table 2). Secondly, the effect of dibutyryl (db)-cAMP, an activator of cAMP-dependent mechanisms, on the elastase release and H_2O_2 production by activated neutrophils was determined. The results revealed that db-cAMP dose-dependently inhibited the fMLP-stimulated elastase release by neutrophils with maximal inhibition of approximately 40 ± 8% seen with 3 mM db-cAMP (n=4). Interestingly, 0.5 mM db-cAMP was sufficient to block the production of H_2O_2 by fMLP-activated neutrophils (n=4). Furthermore, db-cAMP inhibited the elastase release by PMA-activated cells by 23 ± 4% and the production of H_2O_2 by these cells completely (n=3). All values for db-cAMP incubated, fMLP- or PMA-stimulated neutrophils were significantly lower ($p<0.05$) than the values for cells stimulated with fMLP or PMA.

Table 2 Effect of ES on the intracellular cAMP concentration in neutrophils

ES (µg/mL)	cAMP (µM)
0	0.84 ± 0.10
0.5	0.85 ± 0.12
5	1.30 ± 0.14*
50	1.26 ± 0.15*
100	1.44 ± 0.07*

Neutrophils were incubated for 15 sec with ES (range 0.5-100 µg/mL) or no stimulus and then the cAMP content of the samples was quantitated using the Biotrak EnzymeImmunoAssay system. The intracellular cAMP values were calculated from the cAMP contents and the mean cell volume of neutrophils[17]. Results are means ± SEM of four experiments. *Values are significantly ($p<0.05$) different from those for neutrophils not exposed to ES.

Discussion

The main conclusion from the present study is that maggot excretions/secretions inhibit multiple neutrophil pro-inflammatory responses without affecting their antimicrobial functional activities. This conclusion is based on the following observations. First, ES inhibited the release of elastase and production of H_2O_2 by fMLP- and PMA-activated neutrophils in a dose-dependent fashion. These findings with ES are of importance since in chronic wounds the healing process may be impaired by the actions of neutrophils and their products, e.g. proteolytic enzymes and reactive oxygen intermediates, at the surface of wounds[20]. It has been reported that elastase destructs virtually all components of the extracellular matrix[21] and affects epithelial repair mechanisms leading to separation of the dermal and epidermal layers[22]. In light of the notion that excess reactive oxygen

intermediates are responsible for tissue damage[23], it is of interest that neutrophils of patients with chronic venous insufficiency[24] and posttraumatic osteomyelitis[25] are primed to produce high amounts of superoxide anion upon exposure to stimuli. Second, ES inhibited neutrophil chemotaxis towards fMLP in a dose-dependent fashion. In addition, we found that ES reduced the expression of the adhesion molecules CD11b and CD18 on activated neutrophils, indicating that it may modulate adhesion of neutrophils to endothelial cells and subsequently the transendothelial migration process. However, it should be realized that neutrophils also have favorable effects on the wound healing process by their ability to phagocytose and intracellularly kill infectious agents at the affected site. In this connection, we observed that maggot ES did not affect phagocytosis and killing of *Candida albicans* by neutrophils. Notably, maggots aid in the removal of bacteria from wounds by ingesting bacteria together with liquefied necrotic tissue and subsequently killing them in their digestive tract[26]. Third, ES did not induce apoptosis or affect viability of neutrophils, excluding the possibility that ES affect neutrophil responses simply by a cytotoxic effect on the cells.

All neutrophil responses were studied by well-established methods and stimuli. The observation that the maximal inhibitory effect of ES on degranulation and respiratory burst of fMLP-activated neutrophils was greater than on PMA-activated cells may be explained by the differences in the potencies of these two stimuli. Of note, the concentrations of ES used in the present *in vitro* studies are well within the therapeutic range, i.e., those reached on the surface of maggot-treated wounds[2,4]. For instance, the highest concentration of ES, i.e. 100 µg/mL, was obtained after incubating 40-50 maggots in distilled water for 1 h at ambient temperature, and next collecting the fluids. ES in our studies were obtained from sterile maggots under optimal conditions. Unfortunately, the identity of the molecule(s) in ES that is (are) responsible for its inhibitory effects on neutrophil functions remains unknown, and is subject of current studies. Our data indicate that the molecule(s) is (are) heat-labile.

The second conclusion pertains to the mechanisms underlying the inhibitory effects of ES on neutrophil pro-inflammatory responses. The observation that ES did not affect the fMLP-activated rise in the cytoplasmic free calcium concentration in neutrophils indicates that ES act downstream of the diacylglycerol/calcium-mediated activation of protein kinase C. In agreement, ES inhibited PMA-activated neutrophil degranulation and respiratory burst. The finding that fMLP-activated neutrophils were considerable more susceptible to the inhibitory effects of ES than PMA-activated neutrophils indicates that ES may inhibit signaling not only downstream of protein kinase C but also pathways unrelated to protein kinase C activation. Since cAMP-dependent protein kinases are involved in the inhibitory effects of several substances on cells including neutrophils[27,28] and pharmacological activation of this pathway has been reported to inhibit fMLP-activated neutrophil migration, degranulation and the respiratory burst[29,30] we considered the possibility that ES stimulated a rise in the intracellular cAMP concentration in neutrophils. Indeed, ES dose-dependently

induced a transient rise in the intracellular cAMP concentration in neutrophils. In addition, db-cAMP inhibited the fMLP- and PMA-stimulated H_2O_2 production by neutrophils. However, db-cAMP only partly affected the fMLP- and PMA-activated neutrophil degranulation, indicating that ES exerts its inhibitory effects on neutrophil degranulation also by cAMP-independent mechanisms. Although the mechanisms underlying the inhibitory effects of maggot ES on neutrophil pro-inflammatory responses are not fully elucidated, activation of cAMP-dependent mechanisms may be involved.

In summary, the present *in vitro* study shows that maggot excretions/secretions potently inhibit multiple neutrophil pro-inflammatory responses, including chemotaxis, degranulation, respiratory burst and integrin expression without affecting the antimicrobial activities of neutrophils. These inhibitory actions of ES may provide protection against progression towards ongoing inflammation and tissue destruction by neutrophils in chronic wounds.

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