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

Plas, M.J.A. van der

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Cover design by Janus Gjessing

Cover description: ‘Maggot therapy’s modes of action’ in a wound. The wound is placed in a black box. This represents the general use of maggot therapy: maggots are placed on a wound and then the outcome is healing or not. The modes of action -the black box- are unknown by many, but this can be changed by the results described in this thesis.
Different patterns can be observed on the backside of the cover. This symbolises the different processes in wounds that are affected by maggots. The red thread, representing the connection between these processes, is interrupted as not all modes of action are discovered yet.

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Maggot therapy's modes of action

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By this it appears how necessary it is for any man that aspires to true knowledge, to examine the definitions of former authors; and either to correct them, where they are negligently set down, or to make them himself. For the errors of definitions multiply themselves according as the reckoning proceeds, and lead men into absurdities, which at last they see, but cannot avoid, without reckoning anew from the beginning -

Thomas Hobbes, *Leviathan*

Aan mijn ouders
Aan Jens

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CHAPTER ONE

General introduction and scope of this thesis

From the time that we are young and obtain frequent cuts and bruises till later years when trauma, surgery, or illness may result in more extensive tissue damage, we repeatedly go through a series of events leading to repair of injured tissue. In most cases, wounds heal in a seemingly spontaneous manner. In some circumstances, however, the process of wound healing is interrupted and scar formation is delayed. Wound healing is a complex process that can be roughly divided into three overlapping phases: inflammation, proliferation and remodelling (Figure 1). To ensure a positive outcome, wound healing processes are strictly regulated by cell-cell contact and the action of multiple cytokines, chemokines and growth factors released at the site of injury. Unfortunately, wound healing is fragile and subject to failure, and may lead to the formation of chronic wounds. Such wounds may benefit from maggot therapy, i.e. the application of the larvae of certain flies to the open wound. In this thesis, the effects of maggot excretions/secretions on various processes of wound healing are investigated.

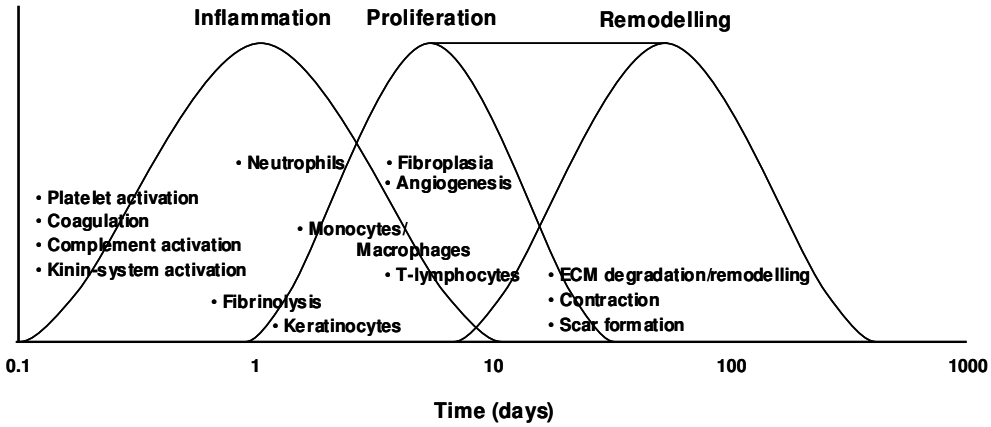

Figure 1 The three phases of wound healing (adapted from S. Enoch and P. Price. Cellular, molecular and biochemical differences in the pathophysiology of healing between acute, chronic and aged wounds. World Wide Wounds, August 2004)

The inflammatory phase

Four major plasma enzyme systems play a role in the control of inflammation: the clotting, kallikrein-kinin, complement and fibrinolytic systems. Following damage to capillary blood vessels an immediate reflex promotes vasoconstriction which slows the blood flow. This enhances platelet adhesion and activation through exposure to thrombogenic components, such as collagen, at the damaged site and leads to the formation of a platelet clot[1,2]. The damaged tissue and activated platelets then produce factors that activate a coagulation signalling cascade leading to the formation of a fibrin clot. The clot contains plasma-derived

glycoproteins, such as fibronectin and vitronectin[3], and plasminogen amongst others, and traps platelets, leucocytes and red blood cells[4]. The blood clot serves as a provisional extracellular matrix allowing cells to migrate into the injured area; fibrin and fibronectin are the most abundant proteins in the provisional matrix. The activated kallikrein-kinin system triggers the release of vasoactive kinins which are involved in vasodilatation and increased vascular permeability[5]. This process resembles that of histamine released from mast cells. The complement system comprises three distinct pathways leading to the formation of factors involved in opsonisation of micro-organisms for ingestion by phagocytes, lysis of micro-organisms, chemotactic attraction of phagocytes, processing of immune complexes and the activation of immune cells[6]. Finally, the fibrinolytic system is responsible for the degradation of fibrin clots and plays a role throughout the different phases of the wound healing process. Hallmarks of fibrinolysis are the formation of plasmin from fibrin-attached plasminogen by plasminogen activators and the subsequent degradation of fibrin by this enzyme[2].

The cellular response to tissue damage starts with the activation of resident cells. Within 24 h of injury, neutrophils are the first to arrive at the injury site in response to chemotactic factors derived from activated platelets, resident (dying) cells and infectious micro-organisms as well as fibrin degradation products and complement factors (C5a and C3a). Efficient recruitment of cells from the circulation into the site involves tethering, rolling and firm adhesion to the endothelial cell surface and finally diapedesis. Regulation of these initial steps involves selectins and integrins that recognize cell surface receptors or matrix proteins such as fibrin, fibronectin and vitronectin[3,7], whereas different molecules are involved in diapedesis (e.g. PECAM and CD99)[8]. Neutrophils are predominant in the first 2-3 days after injury and the numbers peak at around 48 h. Their main function is to eliminate dead cells and micro-organisms by phagocytosis and subsequent destruction in the phagolysosome using oxygen-dependent and -independent mechanisms. The oxygen-dependent mechanisms involve NAPDH oxidases which use molecular oxygen to produce superoxide anions (O_2^-) and which can be converted to hydrogen peroxide (H_2O_2)[9]. The enzyme myeloperoxidase, present in azurophil granules, converts H_2O_2 to hypochlorous acid. The oxygen-independent mechanisms involve degranulation of the granule subsets (azurophil, specific and gelatinase granules and secretory vesicles) into the phagolysosome[10] as well as the extracellular micro-environment. Taken together, the actions of reactive oxygen species and granule contents, including enzymes, antimicrobial peptides and proteins, lead to the destruction of ingested as well as extracellular micro-organisms.

Within 48 h after injury, monocytes are initially attracted to the wound by some of the same chemoattractants that trigger neutrophils. Whereas neutrophil numbers decline after a couple of days, the recruitment of monocytes continues through monocyte-specific chemoattractants[11]. In response to local factors at the wound, monocytes may differentiate to macrophages which then become the predominant cell type later in the inflammatory

phase (around day 5). In addition to clearing the wound of bacteria and tissue debris, monocytes and macrophages in particular regulate the inflammatory process by secreting cytokines, chemokines and growth factors. Initially, monocytes differentiate mainly into pro-inflammatory macrophages. These cells display high levels of pro-inflammatory cytokines and chemokines, which are responsible for the recruitment and activation of additional leucocytes[12] and Th1 lymphocytes. Activation of Th1 cells is further induced by the expression of co-stimulatory molecules on the macrophages and by antigen processing and presentation[13,14]. Th1 lymphocytes activate pro-inflammatory macrophages, thus further enhancing the pro-inflammatory responses. At the end of the inflammatory phase, when most of the infectious agents and tissue debris are cleared, the balance shifts from pro-inflammatory macrophages to macrophages with anti-inflammatory/pro-angiogenic cytokine and growth factor activities. These cells suppress inflammatory responses both directly[15-17] and indirectly by inducing regulatory T cells[18]. They also mediate the clearance of apoptotic cells[16,19] and induce neovascularisation and fibroblast and epidermal cell proliferation[20], thereby playing a pivotal role in the transition from inflammation to repair.

The proliferation phase

The main characteristic of the proliferation phase is the replacement of the provisional matrix with newly formed granulation tissue. This process lasts for about two weeks after wounding.

Granulation tissue formation, the process that ensures reconstitution of the dermis, starts within 4 days after injury. In response to growth factors derived from macrophages and keratinocytes, fibroblasts at the wound edges proliferate and migrate into the provisional matrix, which they then degrade by activation of the fibrinolytic system. In the meantime, secretion of basement membrane components, such as collagen, glycosaminoglycans and glycoproteins such as fibronectin and tenascin[21-23], results in the synthesis of a new collagen-rich matrix, a process termed fibroplasia. Fibroblasts that have migrated to the wound site produce growth factors to further facilitate protein and extracellular matrix (ECM) synthesis. The main function of fibroblasts is the production of new ECM which serves as a scaffold for collagen fibrils and facilitates migration of keratinocytes, fibroblasts and endothelial cells. Binding of these cells to the ECM is mainly facilitated by integrin receptors. The ECM serves also as a reservoir and modulator for (inactive) growth factors[24] and mediates wound contraction[25,26].

To provide nutrients and oxygen to the newly formed granulation tissue, new capillaries/blood vessels are formed by sprouting of pre-existing ones. This process, termed angiogenesis, consists of the activation of endothelial cells by macrophages, degradation of their basement membrane, outgrowth into the wound/new tissue, cell proliferation and migration into the perivascular space, tubule formation, basement membrane reconstitution, formation of new capillary loops and finally re-establishment of the blood flow[27]. There are

several factors that stimulate angiogenesis including growth factors, hypoxic and high-lactate wound environment and low concentrations of reactive oxygen species[28]. Clearly, granulation tissue formation and angiogenesis are overlapping processes. New vessels are essential to support the forming matrix, which in turn supports the new capillary network

Re-epithelialisation is the process of restoring the epidermis and is induced by the presence of several growth factors produced by macrophages, fibroblasts and keratinocytes. Within 24 h after injury keratinocytes start migrating from the wound edges using surface integrin receptors to interact with the provisional matrix while separating eschar and debris that may cover the wound from the newly developing granulation tissue[22]. This process, which involves the degradation of the matrix, is part of the fibrinolytic system. In addition to cleaving plasminogen to form plasmin, plasminogen activators also activate collagenases, which together facilitate the degradation of the ECM and fibrin eschar in the direction of the migration of the cells[1]. To ensure sufficient cell numbers for coverage of the wound, proliferation of keratinocytes located close to the migrating cells is increased while the proliferation potential of migrating keratinocytes is inhibited[29]. When migration ceases, due to contact inhibition, keratinocytes attach to the underlying substratum and differentiate to generate a stratified epidermis.

The remodelling phase

Remodelling occurs throughout the entire wound healing process. The provisional matrix is replaced by granulation tissue which contains type III collagen and newly formed blood vessels, and subsequently is replaced by a collagenous scar predominantly containing type I collagen with less mature blood vessels[26].

Wound contraction is the process that leads to the reduction of the wound area. The degree of contraction depends on the depth of the wound. During granulation tissue formation, fibroblasts undergo phenotypic modulation and differentiate to myofibroblasts, which are characterised by the presence of α-smooth muscle actin fibrils along the plasma membrane. The classical view is that these cells are primarily responsible for contraction by extension of pseudopodia. These facilitate the binding of cytoplasmic actin to extracellular fibronectin and collagen fibres, leading to retraction which draws the collagen fibres to the cell[25,30,31]. However, two contrasting studies reported myofibroblasts are not required for contraction[32,33]. Instead, fibroblasts were shown to influence contraction by reorganizing collagen fibrils rather than pull on the surrounding tissue. Therefore, the mechanisms of wound contraction are unclear and need further exploration.

Approximately 80% (dry weight) of the normal dermis consists of collagen fibres which provide structure, strength and rigidity to the tissue[34]. Within the first week after injury, fibroblasts produce type III collagen to form granulation tissue. However, this collagen is unstructured and does not provide the necessary strength. Therefore, the collagen fibres have to be remodelled and this occurs by degradation of type III collagen and subsequent

synthesis of type I collagen. The degradation of type III collagen depends on the presence of matrix metalloproteinases (MMPs) and their inhibitors produced by macrophages, keratinocytes and fibroblasts in response to cytokines, growth factors and/or cell contact with the ECM[35]. In addition, the newly formed vasculature undergoes remodelling by regression and involution leading to fewer mature vessels[27]. Remodelling continues for up to 2 years but the resulting scar contains fewer cells than normal skin and only reaches up to 70% of its pre-injury strength.

Impaired wound healing

Wound healing is a well-orchestrated but fragile process and is subject to failure to progress through one of its phases (Figure 2), leading to the development of chronic, non-healing wounds. This may result in decreased physical, emotional and social function of patients and therefore a reduced quality of life. In addition, such wounds, which are one of the most common causes of non-traumatic amputation, result in major economic costs for the patients, their families and Society as a whole[36]. Chronic wounds mostly affect people over the age of 60 and the incidence of these wounds is expected to increase.

Impaired healing of wounds can be induced by numerous factors both local and systemic. Local factors include the presence of foreign particles or micro-organisms, ischaemia, tissue maceration, callus formation, pressure and infection whereas systemic factors comprise malnutrition, age, vascular insufficiencies, immune suppressive medication and underlying conditions such as diabetes mellitus. The majority of chronic wounds occur at the lower extremities and can be classified into three categories: venous ulcers, diabetic ulcers and pressure ulcers.

Bacterial infection

Colonisation and infection of the wound surface by bacteria contribute to the failure of wound healing[37-39]. High levels of several bacteria can induce lysis of clots and/or the extracellular matrix[40,41]. This results in impaired cell migration and/or proliferation of leucocytes and fibroblasts, which leads to a delayed immune response. Consequently, bacteria can spread more easily thereby establishing an infection. When leucocytes arrive at the affected site, they initiate a substantial pro-inflammatory response to fight the bacterial infections. However, the bacteria may have formed biofilms, as is often observed in chronic wounds[42]. Due to altered growth characteristics and gene expression profiles[43], these bacteria are protected against the actions of antibiotics[44,45] and cells and effecter molecules of the immune system[39,46]. Stimulation of pro-inflammatory responses therefore continues unabated. In general, bacterial levels above 10^5 organisms/gram of tissue are associated

with poor healing[28]. Clearly, open wounds, especially the presence of necrotic tissue, offer an opportunity for bacterial entry and proliferation.

A large variety of bacterial species have been identified in chronic wounds. Commonly found organisms include gram-positive *Staphylococcus aureus*, *Enterococcus* spp, and *Streptococcus* spp, and gram-negative species such as *Pseudomonas aeruginosa*, *Enterobacter* spp and *Serratia* spp. Furthermore, anaerobic bacteria have also been reported including *Peptoniphilus* spp, *Finegoldia magna* and *Clostridium* spp[47,48].

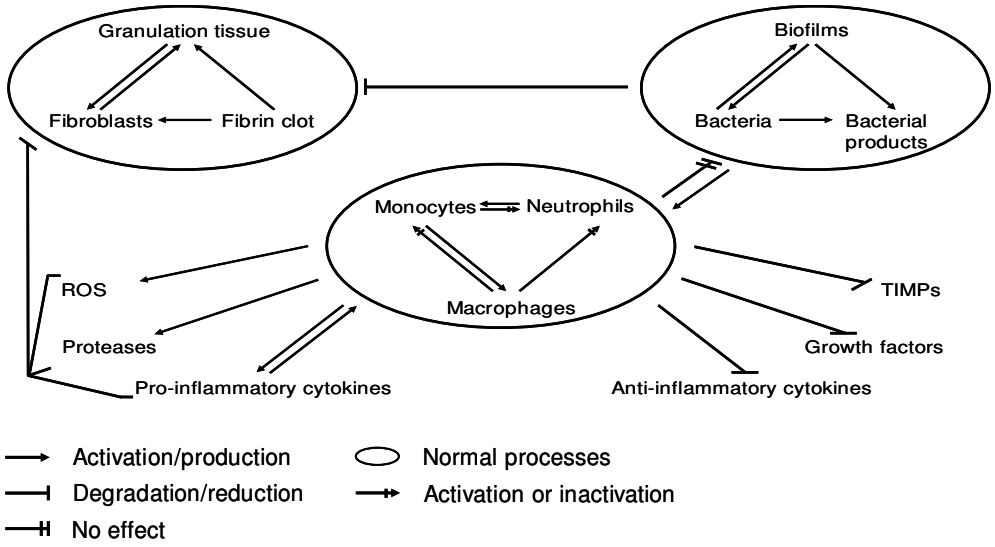

Figure 2 Processes in chronic wounds
ROS, reactive oxygen species; TIMPs, tissue inhibitor of metalloproteinases.

Enhanced inflammatory responses

Although pro-inflammatory responses are essential for wound healing, they become detrimental in wounds where inflammation persists. In the cases where bacteria cannot be eliminated, leucocytes in the wound continue to produce pro-inflammatory mediators. Consequently, the influx of new leucocytes, such as neutrophils, monocytes and macrophages, increases[49-51]. This leads to excessive pro-inflammatory responses in these wounds, which attract even more cells that also produce pro-inflammatory cytokines[52,53]. Phagocytes are activated to release proteolytic enzymes and also to produce large amounts of reactive oxygen species (ROS)[54,55] as a consequence of pro-inflammatory cytokines and/or bacterial products present in the wound. In agreement with this, chronic leg ulcers are associated with elevated expression of pro-inflammatory cytokines, such as TNF-α,

compared to normal healing wounds[56-58]; the levels of these cytokines decrease when the wound begins to heal. Moreover, neutrophils of patients with chronic conditions, such as chronic venous insufficiency[59] and posttraumatic osteomyelitis[60], are primed to produce high amounts of superoxide anion upon exposure to stimuli.

Enhanced proteinase activity

Increased levels of pro-inflammatory cytokines enhance the synthesis and/or release of several matrix metalloproteinases and serine proteases[3,53,61], whereas ROS augment the effects of these proteinases[62,63]. In agreement with this, increased proteolysis has been observed in chronic wounds. Elevated levels of MMP-1, MMP-2, MMP-8 and MMP-9 have been reported for diabetic[64], pressure[65] and venous ulcers[66,67], as compared to normal healing wounds. Altered distribution of proteinase-producing cells in specific wound areas has been observed[68]. In addition, levels of TIMPs are found to be decreased in chronic wounds[64,66]. A possible explanation for this could be that excess levels of proteinases[3,69-71] and ROS[62,63] cause proteinase inhibitor inactivation. Taken together, in chronic wounds the balance between MMPs and TIMPs appears to be disturbed favouring wound degradation. Of note, some contrasting reports have been published[72].

Other proteinases, such as elastase released from azurophil granules, have been reported to be elevated as well in chronic wounds[59,73] due to the large numbers of activated neutrophils. Interestingly, one study showed that elastase degrades MMPs *in vivo* and the authors suggested that elastase is the main cause of ECM destruction[74].

Impaired matrix synthesis and composition

Excess proteinase activities cause destruction of the matrix (and newly formed granulation tissue) by degradation of its components, such as fibronectin, vitronectin and tenascin-C[3,64,75,76]. This leads to impaired cell migration and/or proliferation of fibroblasts, keratinocytes and endothelial cells. Consequently, the mechanical obstruction of re-epithelialisation, wound contraction and remodelling may enhance bacterial infection and prolong the inflammatory response. Moreover, this may lead to the development of fibrin slough and necrotic tissue.

Ulcers may be caused by decreased fibrinolytic activity. In chronic conditions such as obesity and diabetes, levels of plasminogen activator inhibitor 1 (PAI-1) are enhanced[77,78], probably due to elevated levels of inflammatory mediators such as TNF-α and C5a[79,80]. PAI-1 binds to and inactivates plasminogen activators, resulting in impaired lysis of pericapillary fibrin cuffs and subsequent causes ulcer formation[77,81]. In addition, enhanced levels of methylglyoxal are found in diabetic patients resulting in decreased activation of plasminogen activators[82], thereby decreasing fibrinolysis even more. In addition to inducing ulcer formation, decreased fibrinolysis of the provisional matrix has been associated with delayed re-epithelialisation and reduced migration of fibroblasts and keratinocytes[83-86].

Altered growth factor production and/or signalling

Chronic wounds may differ in the levels of growth factors and/or in the cellular responses to these factors from normal healing wounds. It has been reported that growth factors such as PDGF, TGF-β, IGF, FGF and VEGF, which are involved in the recruitment and stimulation of cells that are responsible for repair, are decreased in chronic wounds[87-90]. However, other reports mention no local growth factor deficiency in chronic wounds[57,91,92], whereas increased levels of PDGF and VEGF have been found as well[93,94]. Of note, increased levels of VEGF in chronic wounds were accompanied by increased levels of the VEGF inhibitor[95,96] and/or its degradation[70]. These contrasting results for the levels of growth factors may be caused by differences in wound pathology and cell types or localisation of the cells within the wound and should be further investigated. Furthermore, the mechanisms underlying the imbalances in growth factors and their inhibitors in chronic wounds remain to be elucidated. Excess levels of proteinases[69-71,97] and ROS[62,63] may cause growth factor degradation/inactivation. Furthermore, growth factors may bind to protein macromolecules and become 'trapped' so that they are unable to bind and activate cells[28]. Another possible explanation is that an impaired ECM composition diminishes the actions of growth factors *via* a decrease in integrin binding[3]. Finally, intracellular signal transduction may be dysfunctional in the cells[98].

Changes in cellular profile and activity

Cellular profiles and activities in chronic wounds differ from those in normal healing wounds. Leucocytes produce excessive pro-inflammatory mediators, as indicated above. Pro-inflammatory cytokines have been shown to inhibit proliferation and induce morphological changes in normal skin fibroblasts[99]. These cells become senescent (the process of growing old) as a consequence and have a diminished or even lost the ability to respond to growth factors[100,101]. Additionally, these cells may also be in a state of cell cycle arrest and therefore unresponsive to signalling proteins[102]. In agreement with this, fibroblasts derived from chronic ulcers display a decreased proliferative response to growth factors due to impaired intracellular signalling[98,103,104].

Angiogenesis is impaired in chronic wounds despite enhanced levels of VEGF and, as indicated above, enhanced levels of VEGF inhibitor or degradation of VEGF could be responsible. Another possible explanation is that excess levels of MMPs degrade the ECM thereby impairing endothelial cell migration. In agreement with this, a MMP inhibitor partly restored tubule formation in the presence of chronic wound exudate[105]. However, the effects of factors present in chronic wounds on endothelial cells are unknown.

Finally, epithelialisation is often impaired in chronic wounds, due to impaired migration of keratinocytes. A possible explanation could be the protease-induced degradation of the ECM. Furthermore, impaired signalling, decreased expression of surface receptors

necessary for (growth factor) binding and migration, or increased apoptosis have been proposed[3].

Treatment modalities

Many treatments are available that may induce healing of chronic wounds. These treatments may involve different aspects of wound bed preparation including a restoration of the bacterial balance (e.g. antibacterial agents and dressings), the management of necrosis (debridement), the management of exudate (e.g. dressings, high compression bandaging and vacuum-assisted closure), and the correction of cellular dysfunction and biochemical balances (e.g. growth factors [PDGF-BB], ECM components and bioengineered skin containing fibroblast and/or keratinocytes)[106]. Debridement refers to the removal of damaged, infected and/or dead tissue from the wound bed. Removal of necrotic tissue makes it easier to obtain a moist environment and leads to a better assessment of the wound or ulcer. Furthermore, many bacteria are removed simultaneously, which reduces the bacterial load in the wound. Additionally, debridement removes senescent cells. Besides the clinical relevance, debridement reduces psychological stress due to the bad odour and the appearance of the wound, and it leads to an improved clinical and cosmetic outcome. There are several ways to debride a wound including surgical, mechanical, chemical, enzymatic and autolytic methods. In this thesis maggot debridement therapy is considered in detail.

A history of maggot therapy

Although it has been reported[107] that maggot therapy was used by several peoples such as the aboriginals in Australia, the hill peoples of Northern Burma and possibly the Maya in Central America, the beneficial effects of myasis (maggot infestation) are not universally recognised or appreciated.

Most knowledge about the treatment of wounds in 16th Century Europe is obtained from a book written by Ambroise Paré (1509-1590), chief surgeon to Charles IX and Henri III. In his first 'Journey' he describes a passage from the book 'Of wounds in General' eighth chapter written by John de Vigo stating a frequently applied method to cure wounds made by firearms: *"…to cauterize them with oyl of Elders scalding hot, in which should be mingled a little treacle..."*[108]. In the same period, a surgeon from Turin, who was famous for his treatment of gunshot wounds, used a balm made of new born whelps boiled in the oil of lilies and prepared earthworms with Venetian turpentine[108]. Paré is the first Western surgeon who described human myasis on several occasions. He stated, in reference to a patient with a bad skull wound: *"Now to take away this corruption, I applied at certain times actual cauteries…: but mark, after some months space, a great number of worms came forth by the holes of the rotten bones from underneath the putrified skull…The bone which nature separated was of the bigness of the palm of ones hand… and yet the patient not dye thereof; for he recovered yet beyond all means of expectation"*[109]. Due to this sentence, Paré is regarded by many as one of the first surgeons to recognize the beneficial effects of maggots in the healing of wounds. However, the following two statements make clear that this is not the case: *"what marvail was it, if in these late civil wars, the wounds which were for their quantity small… have caused so many and grievous accidents... Especially, feeling that the Air which encompasseth us, tainted with putrefaction, corrupts and defiles the wounds by inspiration and exspiration… And the corruption was such, that if any changed to be undrest for one day, ... the next day the wound would be full of worms"*[110]. The second statement from Paré, when describing the battle of St. Quintin in 1557 is: *"The wounds of the hurt people were greatly stinking and full of worms with gangrene and putrefaction; so that I was constrained to come with my knife to amputate that which was spoiled. Now their were not any medicines… neither was there half enough to dress so great a number of the people, … and to kill the worms that were entred into their wounds…"*[111].

The first known description of beneficial effects of myasis was recorded by Baron D.J. Larrey (1766-1842), inspector-general of the medical department of Napoleon's army. He wrote: *"The presence of these maggots in the wounds appears to hasten the completion of the suppuration; it caused also an inconvenient itching to the patient, and obliged us to dress the wounds three or four times in the day"*[112]. Further observations of favourable wound myasis, made during the American civil war, came from the confederate surgeons Joseph Jones[113], who investigated the causes of disease and death in confederate prisons

and hospitals, and John F. Zacharias, who is regarded as the first Western physician to intentionally introduce maggots into wounds for debridement (as described in his obituary)[114].

The founder of modern maggot therapy is the orthopaedic surgeon William Baer (1872-1931). During the First World War, Baer treated two soldiers who had lain on the battlefield for seven days. Although having serious injuries, *"…they had no fever and there was no evidence of septicaemia or blood poisoning. … On removing the clothing from the wounded part, much was my surprise to see the wound filled with thousands and thousands of maggots, apparently those of the blow fly. …these wounds were filled with the most beautiful pink granulation tissue that one could imagine"*[115]. In 1928, Baer put his observations into practice by successfully treating his first patients with maggots and, after having some problems with maggot-induced infections, developed a method to sterilize and cultivate the larvae. From 1930 on, maggot therapy became a popular and widespread method for the treatment of infected wounds leading to over 53 publications within the first 5 years[116]. Several reports were published on cheaper ways of capturing, rearing and sterilising maggots as well as on comparing different ways of applying the maggots[117-120], as Baer's method of rearing and sterilising maggots was relatively expensive. In addition, reports were published comparing maggot therapy to several other treatments[121]. Furthermore, the bactericidal activities of maggots[122,123] and the wound healing properties[121,124-126] were investigated thoroughly. Although observations by different researchers led to contrasting conclusions[122,123,126-128], it was generally agreed on that the beneficial effects of maggots were not solely caused by mechanical removal of necrotic tissue but that other factors were involved as well.

In 1935, the results were published of a questionnaire which was sent out to 947 surgeons known to have used maggot treatment both in the USA and Canada[116]. 605 surgeons returned the form leading to a total of 5750 cases; 91.2% of the users expressed a favourable opinion (95.3% of the cases) while the remaining 8.8% was neutral or critical. The major objections of the latter group were the costs of obtaining the maggots, pain and discomfort of patients, along with the time and trouble in applying the treatment. Most research on the use of maggot therapy during this time period was probably published by S.K. Livingston. In 1936, he published a report on the clinical application of maggots and/or maggot 'active principle' in 567 patients[121]. Granulation tissue formation was observed in 88% of the cases leading to hospital discharge. This success rate was 38% higher as compared to other treatments. In addition to maggot treatment, a vaccine therapy was administered intramuscularly consisting of pyogenic organisms suspended in the 'active principle' as a vehicle. However, no results were mentioned and no further references were made about this vaccine therapy possibly due to unfavourable systemic reactions. In 1937, Livingston published another report on the use of 'active principle' of maggots; of the 1020 cases, 415 were treated with living maggots in combination with the active principle while

605 cases were treated by maggot extract alone[129]. The results showed a 60 to 100% clinical improvement, depending on the type of wound, although the extent of improvement differed between the wound types. A year later, Livingston published a preliminary report on the use of a grease-free jelly containing 5% of the 'active principle'; although he reported beneficial effects on healing, no information was given on the consistence of the jelly[127].

At the end of the 1930s, the development of improved surgical techniques and the discovery and distribution of antibiotics made maggot therapy obsolete[107,128,130]. In the following 50 years, maggot therapy was used only as a last resort and became largely forgotten[131]. In 1986, E. Chernin wrote a short review on maggot therapy as he found the 'story of the maggots brief and largely forgotten moment on the surgical stage' worth retelling: *"However unlikely they may seem now as agents of human health, the lowly maggots worked diligently and well. We have since then restored them to their accustomed place as vermin"*[132]. Amusingly, Chernin refers to an article published in 1983 by Pechter and Sherman as *"maggot therapy came and went within a decade or so, though some suggest that the technique may one day reappear"*.

Research into maggot therapy

In the 1930s a large number of experiments were carried out to optimise maggot therapy and also to isolate active components from maggots and their excretions/secretions. Since its re-introduction in the late 1980s and early 1990s, the number of publications dedicated to this therapy has been rising.

Type of flies

Many species of the Diptera family Calliphoridae (blowfly) are capable of infesting living hosts (myasis). These myasis-causing flies can be grouped into two categories: obligate and facultative parasites. Obligate parasites can cause severe damage to healthy tissue as these larvae need living tissue and are therefore unsuitable for maggot therapy. Facultative parasites can feed on living tissue but more commonly use dead/necrotic tissue as their source of nutrition.

Already in the 1930s, practitioners understood the importance of selecting the most suitable fly for maggot therapy. Baer reported the satisfactory use of the blue-black bottle fly *Phormia regina,* the green bottle fly *Sucilia (Lucilia) sericata* and *Lucilia ceaesar*[115]. In addition, Weil *et al* reported that the large blue bottle flies (*Calliphora vomitans* and *C. erythrocephala*) could be used as well[117], whereas Fine and Alexander were more in favour of *Lucilia cuprina*[119]. The most common type of fly used for therapy was *Lucilia sericata* probably because this species was shown to starve on clean granulation tissue[117]. Choosing the right type of fly was not always easy, as noted by Buchman and Blair[117,133]. One of their

batches of larvae bored large cavities in the healthy granulation tissue thereby increasing the size of the wound; instead of using *L. sericata* larvae they probably used the similar looking Texas screw-worm larvae which are obligate parasites[119].

The first commercial supplier of maggots was Lederle (1932), which sold 1000 maggots for five dollars[117,118]. This led to an average treatment cost of 55 dollars per patient. Practitioners searched for ways to rear the larvae cheaper themselves as this was too expensive for many hospitals. For example, Fine and Alexander reported that they obtained their original laying stock by exposing fresh meat in the open near a meat market[117,119]. Nowadays, the maggots of *Lucilia sericata* are used most frequently and are easily available from commercial suppliers such as BioMonde in Germany, Zoobiotic in the United Kingdom and MonarchLabs in the United States. However, hospitals in many countries still rear their own maggots due to costs or transportation problems.

Debridement

The effects of maggots on wounds can be divided into three general mechanisms: debridement, antibacterial effects and stimulation of wound healing.

Debridement is the most known and widely accepted mechanism of action by maggots. This is emphasized by the FDA's 2004 approval of maggots as a medical device to clean out wounds (hence the name maggot debridement therapy). Maggots likely debride wounds by secreting proteolytic enzymes/peptides which dissolve the necrotic tissue. Numerous enzymes have been reported including collagenases[134,135] and serine proteases (trypsin-like and chymotrypsin-like)[136,137], carboxypeptidases A and B, leucine aminopeptidase[138], lipases[139], a metalloproteinase and an aspartyl proteinase[136]. Subsequently, maggots ingest the liquefied tissue which may contain bacteria, cellular debris, and serous drainage of the wound. In addition, the mechanical action of wriggling maggots might enhance debridement, as the maggots probe and macerate the necrotic tissue with their mouth hooks. Together, the secretion of proteolytic enzymes, the ingestion of the resulting liquefied tissue and possibly the mechanical action of maggots result in an efficient debridement of necrotic wounds.

Antibacterial effects

Debridement by maggots results in a wound environment that is less susceptible to bacterial colonisation. Furthermore, as maggots ingest the dissolved tissue, they take up large numbers of bacteria. In 1933, Robinson and Norwood observed that ingested bacteria were abundant in the fore stomach of maggots while the hind stomach showed only slight growth in one-third of the cases[122]. The intestines of the maggots were sterile in all cases[122]. In 2000, similar experiments were performed with a GFP-expressing *E. coli*[140]. The killing of bacteria may be caused partially by proteolytic enzymes which are present in the fore-stomach, but are more abundant in the hind stomach of the maggots as a decrease in

bacterial numbers was seen to be related to the level of enzyme activity[141]. In addition to bacterial killing in the digestive tract, maggots produce antimicrobial molecules in their excretions/secretions (ES). Many reports can be found on the killing of a broad range of microbes by ES including Gram-negative bacteria like *Pseudomonas aeruginosa, Escherichia coli* and *Salmonella* spp, and Gram-positive bacteria such as *Staphyloccus aureus, Staphylococcus epidermis, Listeria monocytogenes* and clinical isolates of MRSA[123,142-145]. Unfortunately, reports either do not mention the amount of ES that was used, or they used very high amounts of ES, i.e. the equivalent of the production by more than 500 maggots in 1 hour. Obviously, the relevance of such amounts of ES would be a subject of debate. Taken together, the ES-induced altered wound pH, the ingestion and subsequent killing of bacteria in the digestive tract of the maggots, and perhaps antibacterial components within ES, could be instrumental in reducing the bacterial load in wounds.

Wound healing

The third effect of maggots on chronic wounds is induction of wound healing. Although many reports describe the appearance of granulation tissue, hardly any research has been published on how maggots induce healing. Chambers *et al* reported that, besides debridement, proteases in maggot ES degrade a variety of ECM components and concluded that enhanced lysis of the ECM could lead to increased healing[136]. Prete *et al* reported enhanced proliferation of fibroblasts in the presence of different maggot preparations[146], whereas Horobin *et al* focused on the effect of maggots on fibroblast adhesion and migration. They reported that ES significantly reduced fibroblast adhesion to both fibronectin and to a lesser extent collagen, due to proteolytic fragmentation of the fibronectin protein surface. Based on these results, it was concluded that fibronectin fragmentation products may activate fibroblasts and enhance their migration[147]. Using a 2D assay, they reported that fibroblast migration across fibronectin is accelerated by serine proteases present in ES[148]. In a later study, they used a 3D assay and showed similar results[149]. In wounds going through the normal phases of healing, these effects of ES would probably enhance healing. However, one of the characteristics of chronic wounds is the presence of enhanced protease activity which disrupts the ECM and the provisional matrix to such an extent that cell migration is impaired. Therefore, degradation of ECM components most likely does not explain the maggot-induced healing. Accordingly, Horobin *et al.* reported that 1 and 5 µg of ES/ml enhanced migration of fibroblasts (both the number of cells and distance covered) while 10 µg of ES/ml actually inhibited migration[149].

Scope of this thesis

In an attempt to obtain insight into the mechanisms underlying the beneficial actions of maggot therapy, we determined the effects of maggot excretions/secretions on several processes involved in wound healing. **Chapter 1** describes the various phases of the wound healing process, the possible mechanisms involved in failure of wound healing and finally the treatment of chronic wounds with medicinal maggots. The healing process in chronic wounds is often complicated by bacterial infections, especially when the bacteria reside in biofilms thus protecting them from the actions of antibiotics and the immune system. We therefore investigated the effects of maggot excretions/secretions (ES) on biofilm formation and breakdown of established *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms. Additionally, the antimicrobial activity of ES against planktonic (free-living) bacteria is described, using two different techniques (**Chapter 2**). There was no killing of bacteria released from the biofilms and we therefore studied the synergistic effects of maggot ES and different antibiotics on the elimination of planktonic *S. aureus,* in both exponential and 'biofilm' phases and describe the effects of these antibiotics on biofilm formation itself (**Chapter 3**).

Although maggots are known primarily for debridement of chronic wounds, little information is available concerning the effects of maggots on haemostatic processes. We therefore investigated the effect of maggot secretions on blood clot formation and on the plasminogen activator induced breakdown of these fibrin clots (**Chapter 4**). Furthermore, we investigated the nature of the active component in the maggot secretions responsible for the observed effects.

The final part of this thesis focuses on the effects of maggot excretions/secretions on inflammatory cells. We report on the effects of ES on the inflammatory responses of neutrophils in reaction to the stimuli fMLP and PMA (**Chapter 5**). For this purpose, we studied chemotaxis, degranulation, H_2O_2-production and phagocytosis and intracellular killing of *Candida albicans* by these cells as well as signal transduction. Besides neutrophils, monocytes also play an important role in fighting invading bacteria. In **Chapter 6** the effects of maggot secretions on the inflammatory responses (cytokine and chemokine production, cell surface receptor expression, chemotaxis, phagocytosis, intracellular killing and signal transduction) of naïve, LPS- or LTA-stimulated monocytes are reported. Monocytes in tissues may differentiate to macrophages with a pro-inflammatory signature or to macrophages with an anti-inflammatory/pro-angiogenic signature; we therefore studied the effects of secretions on monocyte-macrophage differentiation (**Chapter 7**). The main focus of this chapter is on secretions-induced alterations in the regulatory activity of macrophages, using as a read-out the production of cytokines, chemokines and growth factors in response to LPS or LTA. In addition, the expression of various cell-surface receptors was measured.

Finally, the findings from our studies are summarised and discussed in **Chapter 8,** and a summary in Dutch can be found in **Chapter 9**.

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CHAPTER TWO

Maggot excretions/secretions are differentially effective against biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Mariena J.A. van der Plas[1,2], Gerrolt N. Jukema[2], Sin-Wen Wai[1,3], Heleen C.M. Dogterom-Ballering[1], Ellen L. Lagendijk[3], Co van Gulpen[1], Jaap T. van Dissel[1], Guido V. Bloemberg[3,a] and Peter H. Nibbering[1,a]

[1] Department of Infectious Diseases and [2] Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands
[3] Institute of Biology, Leiden University, Leiden, The Netherlands
[a] These authors contributed equally to this study

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Abstract

Objectives: Lucilia sericata maggots are successfully used for treating chronic wounds. As the healing process in these wounds is complicated by bacteria, particularly when residing in biofilms which protect them from antibiotics and the immune system, we assessed the effects of maggot excretions/secretions (ES) on *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms, the clinically most relevant species.

Methods: We assessed the effects of ES on biofilms using microtiter plate assays, on bacterial viability using *in vitro* killing and radial diffusion assays, and on quorum sensing systems using specific reporter bacteria.

Results: As little as 0.2 µg of ES prevented *S. aureus* biofilm formation and 2 µg of ES rapidly degraded biofilms. In contrast, ES initially promoted *P. aeruginosa* biofilm formation, but after 10 h the biofilms collapsed. Degradation of *P. aeruginosa* biofilms started after 10 h and required 10-fold more ES than *S. aureus* biofilms. Boiling of ES abrogated their effects on *S. aureus*, but not *P. aeruginosa* biofilms, indicating that different molecules within ES are responsible for the observed effects. Modulation of biofilms by ES did not involve bacterial killing or effects on quorum sensing systems.

Conclusion: Maggot excretions/secretions are differentially effective against biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Introduction

Chronic wounds cause considerable morbidity and present the health care system with significant costs[1]. Such wounds are common in patients suffering from acute, extended trauma as well as patients with vascular insufficiencies and underlying chronic conditions like diabetes mellitus[2,3] in which even minor wounds become infected and show little tendency to heal. The healing process is often complicated by bacterial infections of the wound surface[4-6]. Bacteria within chronic wounds often reside in biofilms[7] and these bacteria exhibit altered growth characteristics and gene expression profiles as compared with planktonic bacteria[8]. Biofilm formation has been associated with a number of diseases, such as endocarditis[9], cystic fibrosis[10] and osteomyelitis[11]. An important practical consequence of biofilm formation is that the bacteria are protected against the actions of antibiotics[12,13] and cells and effecter molecules of the immune system[6,14]. Moreover, bacterial fragments/ products released from biofilms will continuously attract host immune cells, like neutrophils, to the wound. As these cells cannot remove the infectious cause of inflammation, this will eventually lead to tissue destruction through the actions of bioactive products like reactive oxygen species and proteases released by activated phagocytes[15].

Nowadays, the use of sterile larvae of the green bottle blowfly *Lucilia sericata* in the management of sores, ulcers, and other chronic wounds is becoming increasingly widespread[16-18]. Especially in trauma surgery these maggots can prevent or at least reduce major disabling amputations. Maggots may contribute to wound healing by removing cell debris and non-viable tissue[19], inhibiting the pro-inflammatory responses of phagocytes[20] and promoting tissue remodelling[21]. The molecules involved in these actions are believed to be contained in the excretions/secretions (ES) of the maggots. Interestingly, clinical observations indicated that maggot therapy is more effective in patients with wounds infected with Gram-positive bacteria, like *Staphylococcus aureus,* than those infected with Gram-negative bacteria, like *Pseudomonas aeruginosa*. Additionally, more maggots are needed to accomplish healing of wounds infected with the latter bacterium[22]. Since modulation of bacterial biofilms will have a major impact on the healing process of chronically infected wounds the aim of this study was to investigate the effects of ES on the formation of *S. aureus* and *P. aeruginosa* biofilms and on established biofilms.

Materials and methods

Maggots and maggot excretions/secretions

ES of sterile second- and third-instar larvae of *Lucilia sericata* (a kind gift from BioMonde GmbH, Barsbüttel, Germany) were collected as described[20]. In short, larvae were incubated in water for 60 min. Next, collected ES preparations were checked for sterility and stored at

-20°C. For comparison, we also collected ES according to the method described by Kerridge *et al*[23].

Bacterial strains and growth conditions

Staphylococcus aureus ATCC 29213 (Manassas, VA, USA) were grown in Tryptone Soya Broth (TSB) at 37°C and *Pseudomonas aeruginosa* PAO1[24] in Luria Bertani (LB) medium at 28°C, both under vigorous shaking. The reporter bacteria *Chromobacterium violaceum* CVO26[25] and *Escherichia coli* DH5α strains pAK211[26] and pSB1075[27] were grown in LB medium at 28°C.

Biofilm assay

Biofilm formation of *S. aureus* and *P. aeruginosa* in 96-wells polyvinyl chloride (PVC) plates was conducted as described[28]. In short, bacteria from overnight cultures were diluted with medium 1:1,000 for *S. aureus* and 1:100 for *P. aeruginosa* and 5 µL of these bacterial suspensions were added to each well containing 100 µL of the medium with or without ES (range 0.2-20 µg): the medium for *S. aureus* was 0.5x TSB supplemented with 0.2% (w/v) glucose and for *P. aeruginosa* 0.7x M63. At the indicated intervals, planktonic cells were removed and the wells were washed with tap water. Subsequently, biofilms were exposed to a 1% (w/v) crystal violet solution for 15 min, washed and then incubated in absolute ethanol for 15 min to extract the crystal violet retained by the cells. Next, this solution was transferred to 96-wells plates (Greiner Bio-One, Alphen aan de Rijn, The Netherlands) and used to quantify the amount of biofilm by measuring at OD590 nm. In addition, at various intervals after the start of the experiment, the planktonic cells were harvested and then the bacteria residing in these biofilms were recovered by sonicating three times for 15 s on ice with 30 s between each sonication step. Next, the number of viable bacteria in the suspensions of planktonic cells and of bacteria dispersed from the biofilms was determined microbiologically using serial dilutions of these suspensions plated in six-fold onto COS blood agar plates.

To investigate the effects of ES on established biofilms, we first formed biofilms for 24 h, then the planktonic cells were removed and 100 µL of medium with or without ES (range 0.2-20 µg) were added to the wells.

In vitro killing assay

To further determine the bactericidal effect of ES on planktonic cells, *in vitro* killing assays were conducted as described[29] with minor modifications. Bacteria in mid-log phase were centrifuged at 2,000xg for 10 min, washed with PBS and suspended in 10 mM sodium phosphate buffer (pH 7.4) supplemented with 1% (v/v) TSB to a concentration of $1x10^6$ cells/mL. Subsequently, 200 µL of the bacterial suspension were transferred to Eppendorf

tubes containing vacuum dried ES (range 2-400 µg). After 1 h and 3 h, the number of surviving bacteria was determined microbiologically as described above.

Radial Diffusion Assay (RDA)

To further investigate the antibacterial activity of ES, we used the more sensitive RDA as described[30] with minor modifications. In short, bacteria in mid-log phase were centrifuged at 2,000xg for 10 min and washed with PBS. Next, $1x10^5$ bacteria/mL were dispersed in agar consisting of 1% (w/v) agarose (Sigma-Aldrich, St. Louis, MO, USA) and 1% (w/v) TSB in 10 mM sodium phosphate buffer at 42°C. Subsequently, the agar was poured into petridishes (Greiner Bio-One) and solidified. Next, wells of 3 mm in diameter were made in this agar and 5 µL of vacuum dried ES (range 2-400 µg) solubilised in 0.01% (v/v) acetic acid were transferred to the wells. After 3 h incubation, an overlay agar was poured on top of the bacterial agar. The following day, the diameters of the growth inhibition zones were measured. We validated the assay using 50 µg/mL of human neutrophil peptide 1-3 (hnp1-3) and human lactoferrin-derived peptide (hLF1-11).

Detection of autoinducer activity

Autoinducer activity was measured using the reporter strains *C. violaceum* CVO26 and *E. coli* DH5α containing pAK211 or pSB1075 as described[31]. In short, bacteria were grown overnight in LB medium supplemented with respectively kanamycin (25 µg/mL), chloramphenicol (20 µg/mL) or carbomycin (200 µg/mL). Subsequently, plates were overlaid with top agar existing of LB medium containing 0.8% (w/v) agar (Bacto™agar, BD, Sparks, MD, USA) and 10 µL of the bacterial suspension per mL. Next, 5 µL of vacuum dried ES (range 2-400 µg) solubilised in water or, as a negative control, only water were transferred to the agar and incubated at 28°C for 16 h. As a positive control 0.5 µg of synthetic acyl homoserine lactone autoinducers (kindly provided by Prof. P. Williams, University of Nottingham, UK) was used. Autoinducer activity was detected by the production of a purple pigment (violacein) by *C. violaceum* and by the emission of light when using *E. coli* after applying a Fuji medical X-Ray (Fuji Photo Film Co., Ltd., Tokyo, Japan) on the plates.

Statistical analysis

Results are means ± SEM of at least three experiments using in each experiment two different batches of ES. Differences between the values for ES-exposed and non-exposed bacteria were analyzed using a one-way ANOVA with Dunnett's post-test for multiple comparisons. The level of significance was set at $p<0.05$.

Results

Effect of ES on biofilm formation

To find out if ES can prevent biofilm formation, we determined the amount of biofilm at various intervals after addition of 0-20 µg of ES. The results revealed that after a lag time of 8 h, *S. aureus* started to form a detectable biofilm and that the biofilm formation levelled off after 14 h (Figure 1A). In addition, as little as 0.2 µg of ES completely blocked *S. aureus* biofilm formation. The kinetics of *P. aeruginosa* biofilm formation during the first 24 h were similar to those found for *S. aureus,* but thereafter *P. aeruginosa* biofilms became unstable in several experiments (Figure 1B). Furthermore, enhanced *P. aeruginosa* biofilm formation was seen at 8-10 h after addition of 2 and 20 µg of ES, but thereafter the biofilms formed in the presence of 20 µg of ES, but not 2 µg of ES, collapsed. In agreement, we observed that the number of bacteria in the biofilms exposed to ES for 8-10 h was almost ten-fold higher than in unexposed biofilms (Table 1). Further experiments with higher doses of ES (up to100 µg) revealed that the start of the *P. aeruginosa* biofilm breakdown was dose-dependently enhanced by ES, yet all these biofilms were broken down within 48 h (data not shown). In addition, replacing the medium of biofilms developed in the presence of 20 µg of ES for 8 h with fresh ES-containing medium resulted after 24 h in the breakdown of *P. aeruginosa* biofilms, whereas no breakdown was seen in the wells reincubated with medium alone, indicating that components in ES degraded the biofilms. Of note, *S. aureus* formed biofilms mostly on the bottom of the wells while *P. aeruginosa* formed biofilms on the wall of the

Table 1 The number of bacteria present in the wells of the biofilm formation experiments at 8 and 24 h after starting the experiments.

	Biofilm		Planktonic cells	
ES (µg/mL)	0	20	0	20
S. aureus				
t = 8 h	3.2 ± 1.7 (x 10^6)	no	4.2 ± 0.6 (x10^7)	3.8 ± 0.8 (x10^7)
t = 24h	6.7 ± 1.1 (x 10^6)	no	3.8 ± 0.8 (x10^7)	5.1 ± 0.5 (x10^7)
P. aeruginosa				
t = 8 h	7.0 ± 1.2 (x 10^5)	5.4 ± 2.6 (x 10^6)*	1.6 ± 0.7 (x 10^7)	1.9 ± 1.1 (x 10^7)
t = 24h	2.9 ± 1.0 (x 10^7)	no	4.0 ± 2.4 (x 10^8)	4.4 ± 2.0 (x 10^8)

Results are means ± SEM of 4-6 experiments. 'no' indicates that no biofilm was detectable. *Significant ($p<0.05$) differences between the values for bacteria exposed to ES and those for non-exposed bacteria.

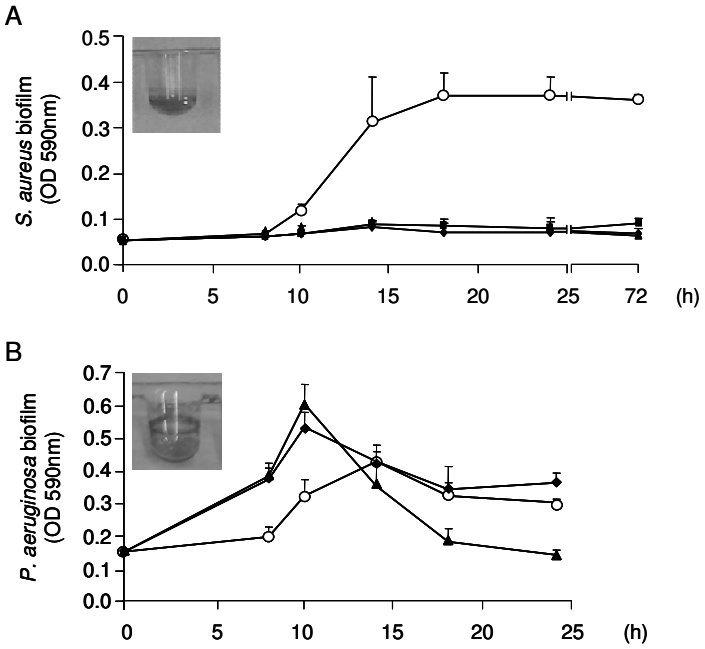

Figure 1 Effect of maggot excretions/secretions on biofilm formation by *S. aureus* (A) and *P. aeruginosa* (B). Results are means ± SEM of 4-5 experiments. Open circles = no ES; filled squares= 0.2 µg of ES; filled diamonds = 2 µg of ES; filled triangles = 20 µg of ES.
A: From 10 h on, all values are significantly ($p<0.05$) different from those for biofilms without ES.
S. aureus mainly formed biofilms at the bottom of the wells (insert).
B: Values for 20 µg of ES are significantly higher at 8 h and 10 h, and significantly lower at 18 h and 24 h than those for biofilms without ES. *P. aeruginosa* formed a ring on the wall of the wells at the air-liquid interphase (insert).

Table 2 Effect of heat-treatment on the activity of 20 µg of ES against biofilms.

	Treatment		
	no ES	native ES	boiled ES
S. aureus			
Biofilm formation	0.37 ± 0.04	0.09 ± 0.01	0.29 ± 0.03*
Biofilm breakdown	0.38 ± 0.07	0.10 ± 0.06	0.46 ± 0.07*
P. aeruginosa			
Biofilm formation	0.29 ± 0.01	0.15 ± 0.02	0.12 ± 0.02
Biofilm breakdown	0.42 ± 0.03	0.22 ± 0.06	0.16 ± 0.01

Results are means ± SEM of 3-5 experiments. *Significant ($p<0.05$) differences between the values for biofilms exposed to boiled ES and those to native ES.

wells at the air-liquid interphase (Figure 1 A,B inserts). Interestingly, treatment of 20 µg of ES for 2 h at 100°C completely abrogated the effects on *S. aureus* biofilm formation, but not on *P. aeruginosa* biofilm formation (Table 2), indicating that different molecules within ES modulate *S. aureus* and *P. aeruginosa* biofilm formation.

Effect of ES on established biofilms

Next, we determined the effects of ES on established biofilms. The results showed that within 2 h after addition of ES the amount of *S. aureus* biofilm was dose-dependently reduced and a complete breakdown was seen with 2 and 20 µg of ES (Figure 2A). Furthermore, 0.2 µg of ES gradually reduced the amount of biofilm within the first 6 h and thereafter the amount of biofilm remained constant. Established *P. aeruginosa* biofilms were initially stimulated by ES and after 10 h gradually broken down by 20 µg of ES, while 2 µg of ES did not cause an effect (Figure 2B). Heat treatment of ES completely abrogated their effects on established *S. aureus* biofilms, but not on established *P. aeruginosa* biofilms (Table 2).

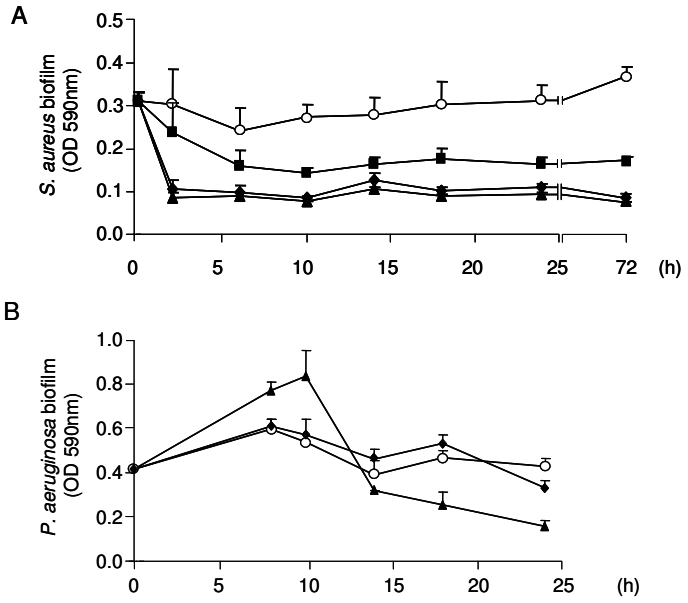

Figure 2 Effect of maggot excretions/secretions on established biofilms of *S. aureus* (A) and *P. aeruginosa* (B).
Results are means ± SEM of 5-6 experiments. Open circles = no ES; filled squares = 0.2 µg of ES; filled diamonds = 2 µg of ES; filled triangles = 20 µg of ES.
A: All values of 2 and 20 µg ES are significantly ($p<0.05$) different from those for biofilms without ES. From 10 h on, 0.2 µg of ES are significantly different from those for biofilms without ES.
B: Values for 20 µg of ES are significantly higher at 8 h and 10 h, and significantly lower at 18 h and 24 h compared to biofilms without ES.

Effect of ES on bacterial viability

Since ES may have bactericidal activities against Gram-positive and Gram-negative bacteria,[23,32] we determined the effect of ES on the number of viable biofilm-associated and planktonic *S. aureus* and *P. aeruginosa* in our biofilm experiments. The results revealed that at the current doses and conditions ES did not kill planktonic bacteria (Table 1). In addition, the total number of bacteria in the wells was not significantly altered indicating that ES did not disrupt biofilms simply by killing bacteria. Furthermore, 20 µg of ES were not bactericidal against *S. aureus* and *P. aeruginosa* in *in vitro* killing and radial diffusion assays. *In vitro* killing experiments revealed that only the largest dose of ES studied (400 µg) reduced the number of viable *S. aureus* after 3 h by 73 ± 10%, but not after 1 h, as compared with the control (n = 7). Using RDAs we found that ES killed *S. aureus* in a dose-dependent fashion with as little as 40 µg of ES being effective (Figure 3). Heat-treatment abolished the bactericidal effects of ES on *S. aureus* in the *in vitro* killing assays and it reduced the effects in the RDAs by 79 ± 16% (n = 4). In contrast, ES (up to 800 µg) did not reduce the number of viable *P. aeruginosa*. Finally, no differences in the antibacterial activity between ES preparations obtained by the method of Kerridge *et al* [23] and our ES preparations were noted.

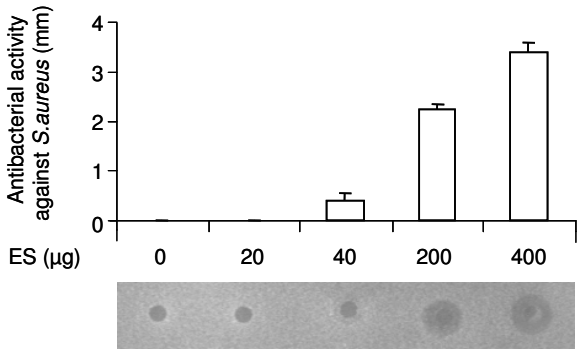

Figure 3 Antimicrobial activity of maggot excretions/secretions against *S. aureus* using a radial diffusion assay. Results are means ± SEM of 6 experiments. The diameter of the clearance zone was corrected for the diameter of the well.

Effect of ES on quorum sensing systems of Gram negative bacteria

As quorum sensing systems control bacterial functions, such as biofilm formation[33], interference with these bacterial systems could explain the effects of ES on biofilms. Therefore, we determined the ability of ES to mimic or antagonize the actions of various *N*-acyl homoserine lactones (AHLs) using specific reporter bacteria. The results showed that

ES (0.2-200 µg) had neither mimicking nor antagonizing effects on quorum sensing systems detecting short chain (C6/C8) AHLs, as assayed with the reporter bacteria *C. violaceum* CVO26 and *E. coli* DH5α containing pAK211. The positive control (synthetic C6 AHLs) showed zones of approximately 5 cm in both systems (n = 3). Furthermore, ES had no effect on quorum sensing systems responding to long chain (C10/C12) AHLs assayed in *E. coli* DH5α containing pSB1075; the positive control (synthetic C10 AHLs) caused a zone of 5 ± 0.4 cm (n = 3).

Discussion

The main conclusion from the present study is that maggot excretions/secretions are differentially effective against biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. This conclusion is based on the following observations. First, *S. aureus* biofilm formation was blocked by as little as 0.2 µg of ES per well, whereas 2 µg of ES per well was sufficient to degrade established biofilms within 2 h. Secondly, *P. aeruginosa* biofilm formation was initially enhanced by ES and after 10 h biofilms treated with 20 µg of ES, but not 2 µg of ES, degraded and during the remaining period of the analysis no biofilms could be detected. Interestingly, others reported similar effects of the prokaryotic predator *Micavibrio aeruginosavorus* on *P. aeruginosa* biofilm formation and suggested that increased cell-cell interactions may explain the initial enhancement of biofilms[34]. Thirdly, the doses of ES used in this study were within the therapeutic range, i.e., those present at the surface of maggot-treated wounds[35,36]. For instance, 20 µg of ES were obtained after incubating approximately 10 maggots in distilled water for 1 h. It should be realized that in our *in vitro* experiments ES were added only once to the bacteria and/or bacterial biofilms, whereas in wounds, maggots are continuously present. Furthermore, ES were obtained from sterile maggots. Since it is likely that ES of maggots exposed to bacteria in a wound have an altered composition, it is of interest that ES obtained from bacteria-exposed maggots were as effective against bacterial biofilms as sterile ES (MJA van der Plas *et al*, unpublished observations).

The second conclusion pertains to the mechanism(s) underlying the prevention of biofilm formation and the breakdown of bacterial biofilms by ES. The possibility that ES modulate biofilms simply by killing the bacteria is highly unlikely since in our biofilm experiments ES did not affect the number of viable bacteria in the wells. However, it is reported by several groups[23,32,37] that ES have bactericidal properties against planktonic bacteria, although the used amounts are not within the therapeutic range or not mentioned at all. Therefore, we decided to investigate the bactericidal activity further by using two different methods described in these reports; the RDA being the most sensitive assay but the *in vitro* killing assay resembling the biofilm experiments more. In agreement with our biofilm data, *S.*

aureus were not killed at the biofilm-effective amounts of ES while *P. aeruginosa* was not killed at all. Investigation into the effects of ES on quorum sensing signalling pathways in several Gram-negative reporter strains showed that ES do not mimic or antagonize short and long chain *N*-acyl homoserine lactones. However, these data do not exclude the possibility that ES interfere with quorum sensing signalling of bacteria in the wound. Although no definitive explanation for the differences in effects of ES on *S. aureus* and *P. aeruginosa* biofilms can be offered on the basis of our data, we concluded that the observed effects are mediated by different molecules and mechanisms, since heat-treatment completely abrogated the effects of ES on *S. aureus*, but not on *P. aeruginosa*, biofilms. This suggests that proteins or heat sensitive peptides within ES may be responsible for the breakdown of *S. aureus*, but not of *P. aeruginosa* biofilms. More research, including purification of these compounds, is needed to gain a detailed understanding of the mechanisms involved in the modulatory effects of ES on biofilms.

We are the first to report that ES disrupt bacterial biofilms. It should be kept in mind that we required more ES to disrupt *P. aeruginosa* biofilms than *S. aureus* biofilms and that low doses of ES can result in enhancement of *P. aeruginosa* biofilms. In addition, it has been shown *in vitro* that *P. aeruginosa,* but not *S. aureus,* impairs maggot survival[38]. Together, these data are in agreement with clinical findings[22] indicating that more maggots should be used for wounds infected with *P. aeruginosa* (compared to *S. aureus*). Furthermore, as a result of biofilm breakdown, the bacteria become susceptible to actions of antibiotics and the immune system as well as to actions of maggots[39]. Therefore, ES (especially in combination with antibiotics) are a very promising source of candidates for the development of new treatments for biofilm-associated diseases, including cystic fibrosis, infected medical devices, like catheters and prosthesis, and chronic wounds.

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CHAPTER THREE

Synergistic actions of maggot excretions/secretions and antibiotics against biofilm-associated *Staphylococcus aureus*

Mariena J.A. van der Plas[1,2], Cheryl Dambrot[1], Heleen C.M. Dogterom-Ballering[1], Simone Kruithof[1], Jaap T. van Dissel[1], and Peter H. Nibbering[1]

[1] Department of Infectious Diseases and [2] Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands

Submitted

Abstract

Objectives: Maggots of the blowfly *Lucilia sericata* are used for the treatment of chronic wounds. Earlier we reported that maggot excretions/secretions (ES) breakdown *Staphylococcus aureus* biofilms but do not kill the bacteria. As many antibiotics are not effective against biofilms and the associated bacteria we assessed the effect of combining ES and antibiotics on *Staphylococcus aureus* biofilms and on the survival of the bacteria released from the biofilms.

Methods: Combinations of ES and vancomycin, daptomycin or clindamycin on *S. aureus* ATCC 29213 biofilms and bacterial viability were tested using microtitre plates and *in vitro* killing assays.

Results: Vancomycin and daptomycin dose-dependently enhanced biofilm formation, whereas clindamycin reduced *S. aureus* biofilm size. Adding ES to antibiotic incubations caused a complete biofilm breakdown. There was a lag time before bacteria released from biofilms became susceptible to vancomycin and clindamycin, which was also dependent on refreshing medium and allowing time to restart bacterial replication. Daptomycin showed direct activity against biofilm-derived bacteria. In exponentially growing bacteria, ES did not affect the bactericidal activity of antibiotics whereas ES increased the activity of daptomycin against bacteria upon release from the biofilms.

Conclusions: Maggot excretions/secretions release biofilm-associated *S. aureus* into the surrounding milieu allowing them to become exposed to the action of antibiotics; the effect depends on the pharmacodynamic property of the specific antibiotic drug.

Introduction

Chronic wounds are common in patients with vascular insufficiencies and underlying chronic conditions such as diabetes mellitus, as well as patients suffering from acute, extended trauma[1,2]. These wounds and consequent amputations result in decreased physical, emotional and social function of patients, a reduced quality of life and major economic costs for patients, their families and society[3,4]. A severe complication of the healing process is bacterial colonization and subsequent infection of the wound surface[5-7], especially when the bacteria are residing in biofilms[8]. These bacteria exhibit altered growth characteristics and gene expression profiles, as compared to those present freely in the environment, the so called planktonic bacteria[9]. Importantly, biofilm formation and the consequences thereof for bacterial growth characteristics render microorganisms resistant to the action of many antibiotics[10,11] as well as cells and effecter molecules of the host's immune system[7,12]. Bacterial fragments/products released from biofilms continuously attract host cells to the wound. As phagocytes cannot ingest the biofilm-associated bacteria and therefore are unable to eliminate the cause of infection, the subsequent accumulation of inflammatory cells and enhanced release of pro-inflammatory cytokines, proteases and reactive oxygen species eventually lead to inactivation of growth factors and tissue destruction[13,14] thereby contributing to the establishment and/or maintenance of chronic wounds.

Sterile larvae -maggots- of the green bottle blowfly *Lucilia sericata* are used as a treatment of various types of chronic wounds[15-17]. Earlier we reported maggot excretions/secretions (ES) to breakdown *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms[18]. However, the bacteria released from these biofilms were not killed by ES. On the other hand, many antibiotics cannot break down bacterial biofilms but effectively kill planktonic bacteria. Therefore, we assessed the effect of combinations of maggot ES and antibiotics on *Staphylococcus aureus* biofilms and on the survival of the bacteria released from these biofilms.

Materials and methods

Maggots and maggot excretions/secretions

ES of sterile second- and third-instar larvae of *Lucilia sericata* (a kind gift from BioMonde GmbH, Barsbüttel, Germany) were collected as described[19]. In short, larvae were incubated in H_2O for 60 min. Sterile ES preparations were harvested, pooled and stored at -20°C.

Antibiotics

Vancomycin (Pharmachemie B.V., Haarlem, The Netherlands), daptomycin (Cubicin, Chiron Corporation Limited, Uxbridge, UK), and clindamycin (Upjohn GmbH, Heppenheim, Germany) were dissolved in distilled water to a final concentration of 10 mg/mL.

Staphylococcus aureus cultures

Staphylococcus aureus ATCC 29213 (Manassas, VA, USA) were grown in Tryptone Soya Broth (TSB) at 37°C under vigorous shaking. The MIC-values for this strain are 0.5-2 µg/mL for vancomycin, 0.25-1 µg/mL for daptomycin and 0.06-0.25 µg/mL for clindamycin[20].

Biofilm assay

Biofilm formation of *S. aureus* in 96-wells polyvinyl chloride (PVC) plates was conducted as described[18]. In short, bacteria from overnight cultures were diluted 1:1,000 and 5 µL of these bacterial suspensions were added to each well containing 100 µL of 'biofilm medium' consisting of 0.5x TSB supplemented with 0.2% (w/v) glucose. After 24 h, planktonic cells were removed and 100 µL of biofilm medium with or without antibiotics (1-400 µg/mL) and/or ES (20-200 µg/mL) were added to the biofilms. At the indicated time intervals, planktonic cells were harvested from these wells and the numbers of viable bacteria were determined microbiologically using serial dilutions of these suspensions plated in six-fold onto agar plates. The reliable lower detection limit of this method is 100 cfu/well. In addition, after washing the wells with tap water, biofilms were exposed to a 1% (w/v) crystal violet solution for 15 min, washed and then incubated in absolute ethanol for 15 min to extract the crystal violet retained by the cells. Next, this solution was transferred to 96-wells plates (Greiner Bio-One, Alphen aan de Rijn, The Netherlands) and used to quantify the amount of biofilm by measuring the absorbance at 590 nm.

Furthermore, we investigated the effect of antibiotics on bacteria derived from the biofilms and subsequently transferred to fresh biofilm medium. For this purpose, the planktonic cells were removed from 24 h old biofilms and fresh biofilm medium was added to the wells containing ES (20 - 200 µg/mL) or H_2O as a control. After an additional 24 h, the bacteria released from the biofilms were harvested and 25 µL of these bacterial suspensions were transferred to wells of a PVC plate containing 75 µL of TSB medium supplemented with antibiotics; the final concentrations of the medium was 0.5 x TSB and 0.2% glucose. After 3 h and 24 h, the numbers of surviving bacteria were determined microbiologically as described above.

Influence of maggot ES on the concentration-effect relationship for antibiotics on exponential growing S. aureus

To further determine the concentration-effect relationship for antibiotics on planktonic *S. aureus* in the presence or absence of ES, *in vitro* killing assays were conducted as described[21] with minor modifications. Bacteria in mid-log phase were centrifuged at 2,000xg for 10 min, washed twice with PBS and resuspended in biofilm medium supplemented with antibiotics (0.005 - 500 µg/mL) and/or ES (20 - 200 µg/mL) to a concentration of 1 x 10^7 bacteria/mL. Subsequently, 100 µL aliquots of these bacterial suspensions were transferred

to wells of a 96-wells PVC plate and incubated at 37°C. After 1, 2 and 3 h, the numbers of surviving bacteria were determined microbiologically as described above.
Next, the differences between the logarithms (base 10) of the numbers of CFU in the absence and presence of antibiotics and/or ES were calculated for each time point[22]. For further calculations, the highest value of the net killing rate during the 3 h of exposure was used (E_R). The concentration-effect relation was established by using the Hill Equation:

$$E_R = E_R,max \times C / (EC_{50} + C)$$

where E_R,max is the estimated maximal killing rate, C the antibiotic concentration (µg/mL), and EC_{50} the estimated antibiotic concentration at which 50% of the maximal killing is reached. The parameters of this pharmaco-dynamic model were calculated in SPSS using non-linear regression analysis.

Statistical analysis
Statistical analyses were performed using Graphpad Prism version 4.02. Statistical differences between the values for ES-incubated and control-incubated bacteria were analyzed using a paired t-test. The level of significance was set at p-values < 0.05.

Results

Effect of antibiotics and ES on S. aureus biofilms
Planktonic cells from 24 h biofilms were removed and mixtures of fresh biofilm medium containing antibiotics (1 – 400 µg/mL) were added to the wells for 3 h or 24 h.
The results showed a dose-dependent increase in biofilm size by vancomycin and daptomycin already within 3 h (Table 1). This effect persisted over the next 21 h. In contrast, clindamycin dose-dependently decreased the amount of biofilm; after 3 h of incubation the biofilm partly vanished although total breakdown was not observed (Table 1). As reported earlier[18] within 3 h ES degraded the *S. aureus* biofilms completely and this effect was not counteracted by any of the antibiotics (data not shown).

Effect of combining ES and antibiotics on the viability of S. aureus released from biofilms
To investigate whether the antibiotics eradicate the bacteria released from the biofilms and if ES influence this process, planktonic cells were harvested from the wells and their viability tested. Preliminary experiments revealed no reduction in the number of viable bacteria when using ≤10 µg/mL of vancomycin and daptomycin.

Table 1 Effect of antibiotics on established biofilms of *Staphylococcus aureus*

	Vancomycin		Daptomycin		Clindamycin	
µg/mL	3 h	24 h	3 h	24 h	3 h	24 h
0	0.18 ± 0.02	0.30 ± 0.02	0.21 ± 0.01	0.30 ± 0.02	0.21 ± 0.01	0.29 ± 0.02
1	0.19 ± 0.03	0.26 ± 0.04	0.20 ± 0.03	0.27 ± 0.03	0.20 ± 0.03	0.24 ± 0.04
5	0.21 ± 0.03	0.29 ± 0.05	0.20 ± 0.03	0.26 ± 0.05	0.17 ± 0.03	0.24 ± 0.04
10	0.27 ± 0.04*	0.38 ± 0.06*	0.23 ± 0.04	0.35 ± 0.07	0.17 ± 0.04	0.24 ± 0.04
50	0.26 ± 0.04*	0.36 ± 0.05*	0.26 ± 0.04*	0.45 ± 0.10*	0.16 ± 0.01*	0.24 ± 0.01*
100	0.26 ± 0.04*	0.40 ± 0.03*	0.27 ± 0.01*	0.39 ± 0.03*	0.16 ± 0.01*	0.22 ± 0.01*
200	0.26 ± 0.04*	0.35 ± 0.02*	0.28 ± 0.02*	0.36 ± 0.03*	0.16 ± 0.01*	0.21 ± 0.02*
400	0.22 ± 0.01*	0.43 ± 0.03*	0.33 ± 0.02*	0.38 ± 0.04*	0.15 ± 0.01*	0.21 ± 0.02*

Results (Abs at 590 nm) are means ± SEM of ≥ 4 experiments. *Values are significantly (*$p<0.05$) different from those for control biofilms. For all samples, the addition of ES resulted in total breakdown of the biofilms (Abs < 0.10).

Vancomycin at concentrations of 50 µg/mL or higher significantly reduced the number of viable bacteria by 24 h (Figure 1A), but not yet after 3 h (data not shown). Daptomycin dose-dependently reduced the number of biofilm-derived bacteria within 3 h, up to 99.9 ± 0.02% at a concentration of 400 µg/mL (data not shown). This reduction in bacterial numbers continued the next 21 h (Figure 1B). After 3 h of incubation, the number of viable bacteria was 90% lower in the presence of clindamycin compared with control incubations of bacteria derived either from ES-treated or untreated biofilms (data not shown). The following 21 h, no bacterial outgrowth was observed in the presence of clindamycin (Figure 1C). Furthermore, a dose-dependent effect of clindamycin was observed at the lowest concentrations used in the experiments (i.e., 1, 5 and 10 µg/mL, resulting in a reduction of viable bacteria by 53 ± 9%, 78 ± 4% and 80 ± 14%, respectively), whereas at clindamycin concentrations above 10 µg/ml a maximal inhibition had been reached.

Of note, at all conditions chosen, ES (200 µg/mL) did not affect the antibiotic-induced bacterial killing. Using 20 µg of ES/mL yielded identical results (data not shown).

Effect of ES and antibiotics on biofilm-derived bacteria transferred to fresh biofilm medium

As large numbers of bacteria derived from the biofilms remained viable in the presence of antibiotics, we considered the possibility that this resistance was caused by phenotypic variation. Therefore, bacteria were transferred from ES-incubated or control-incubated biofilms to fresh biofilm medium supplemented with antibiotics.

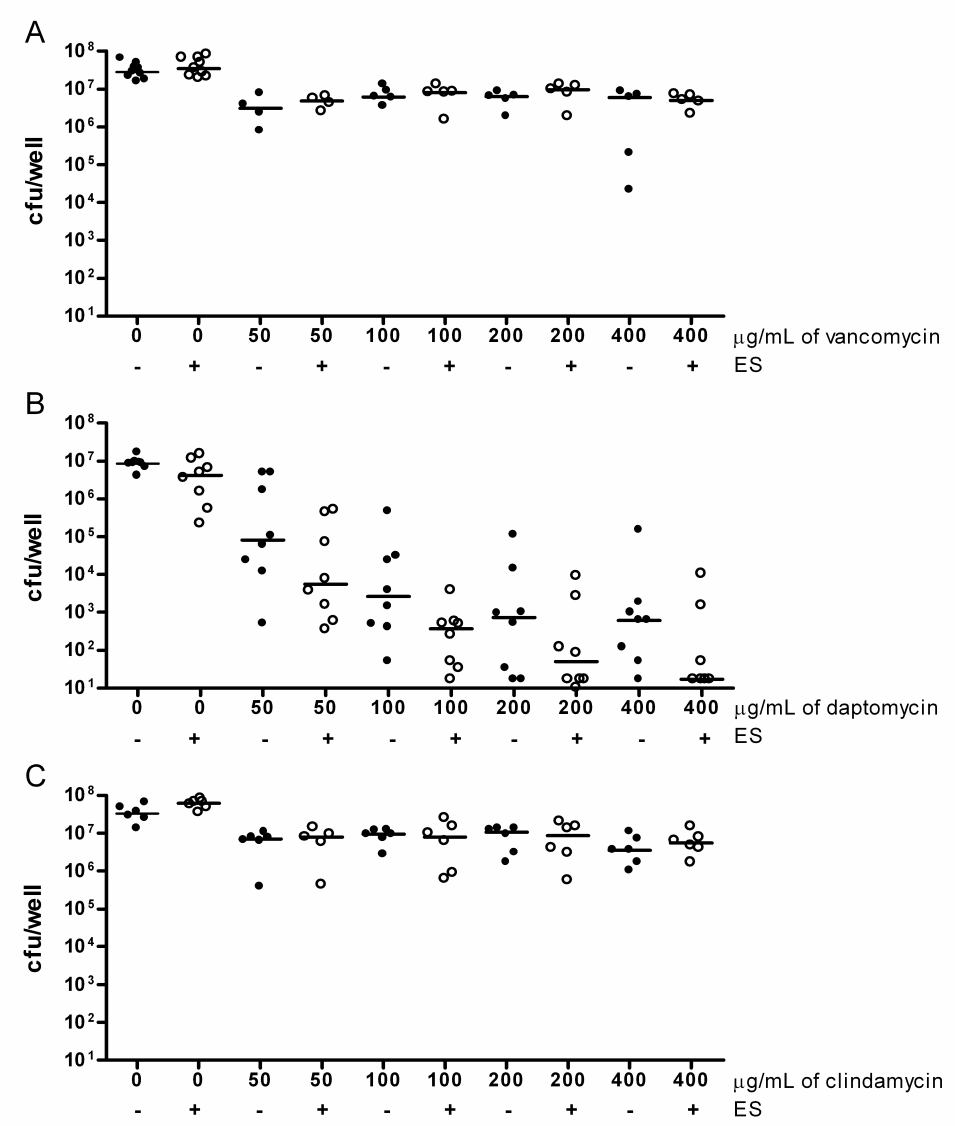

Figure 1 Effect of antibiotics and/or ES on biofilm-derived *Staphylococcus aureus*.
Biofilms were incubated with increasing concentrations of vancomycin (A), daptomycin (B) or clindamycin (C) in the absence or presence of 200 µg of ES/mL for 24 h. Results of 4-6 experiments are shown with the median (line). The values from the antibiotic samples are significantly different from the control wells.

Vancomycin failed to affect the number of viable bacteria at 3 h but reduced the number by 99% at 24 h. This effect was independent of the chosen dose of antibiotics or the presence of ES (Figure 2A). Daptomycin dose-dependently reduced the number of bacteria within 3 h. This antimicrobial effect was further enhanced by 200 µg of ES/mL (Figure 2B) but not by 20

µg of ES/mL (data not shown). After 24 h, all bacteria were killed by the used concentrations of daptomycin independent of the presence of ES (data not shown). Clindamycin prevented outgrowth of the bacteria at 3 h of incubation at all antibiotic concentrations used (data not shown) and this effect remained constant during the following 21 h (Figure 2C); ES did not affect the activity of clindamycin on the bacteria.

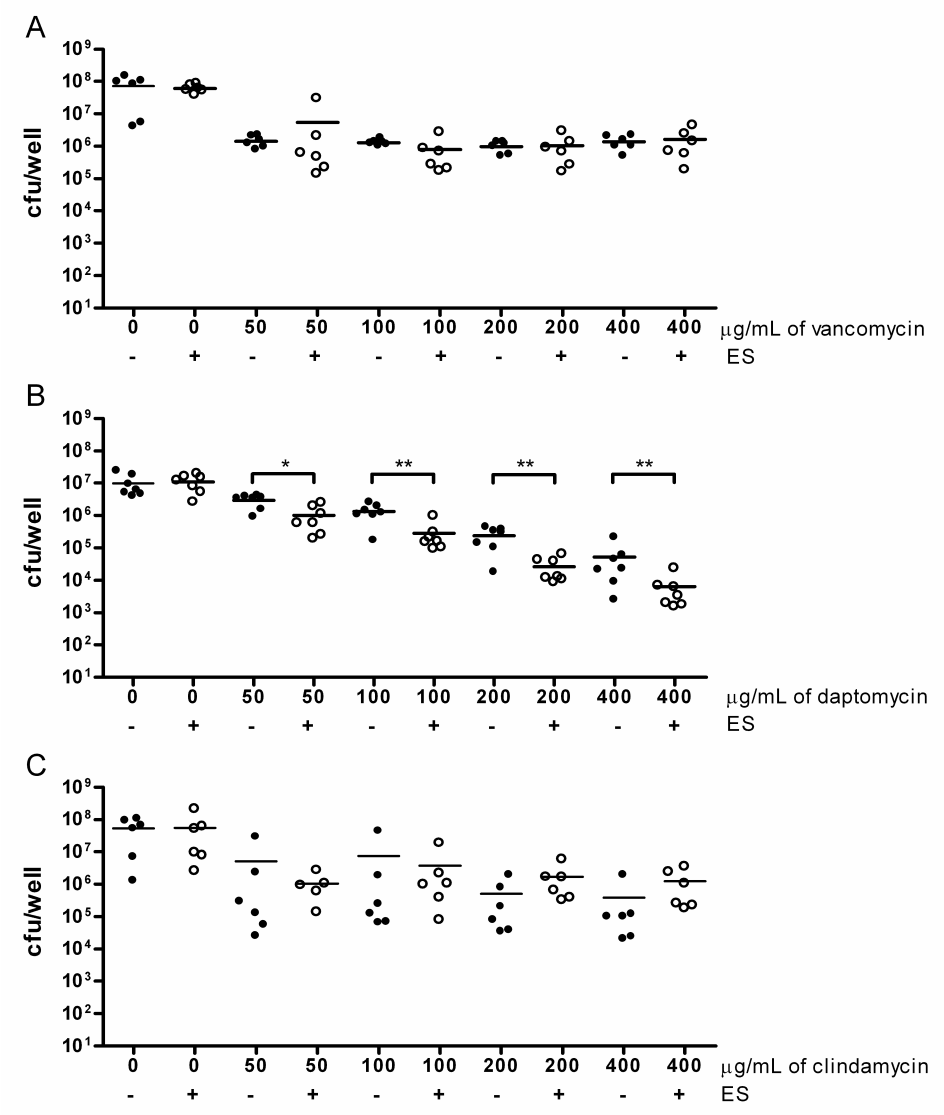

Figure 2 Effect of antibiotics on *Staphylococcus aureus* derived from ES-incubated and control-incubated biofilms. Results of 6-7 experiments with the median (line) are shown after 24 h incubation for vancomycin (A) and clindamycin (C) whereas the effect of daptomycin (B) was obtained after 3 h. Values are significantly (*$p<0.05$ and **$p<0.005$) different from those for bacteria derived from control-incubated biofilms supplemented with antibiotics.

Effect of ES on the concentration-effect relationship of antibiotics on exponentially growing S. aureus

To investigate the activity of the antibiotics on exponentially growing bacteria, we determined the growth curves at various antibiotic concentrations using the Hill equation. The results showed a dose-dependent reduction of viable *S. aureus* through the actions of all three antibiotics, although the maximum effect of daptomycin was higher than that of clindamycin and vancomycin, which were equally effective against the bacteria (Figure 3 A-C). The estimated EC_{50} and E_R, max values are given in Table 2. Additionally, we determined the effect of ES on these parameters of the antibiotics. The effects of vancomycin (Figure 3A) and clindamycin (Figure 3C) on exponential growing *S. aureus* were not significantly affected by ES although the E_R,max was enhanced somewhat and the EC_{50} decreased (Table 2). The E_R,max of daptomycin was enhanced in the presence of both 20 µg and 200 µg of ES/mL, although this effect did not reach a level of significance except for incubation with 100 µg of daptomycin (Figure 3B). Of note, 500 µg of daptomycin/mL was sufficient to kill all bacteria within 1 h in 4 out of 5 experiments under all conditions. Furthermore, the maximum effect of clindamycin was observed at 1 µg/mL, that of vancomycin at approximately 10 µg/mL, whereas of daptomycin 500 µg/mL was required to reach a maximal effect.

Table 2 Pharmacodynamic parameters of the antibiotics and ES

	Clindamycin		Vancomycin		Daptomycin	
ES (µg/mL)	E_R,max/h	EC_{50} (µg/mL)	E_R,max/h	EC_{50} (µg/mL)	E_R,max/h	EC_{50} (µg/mL)
0	1.64	0.036	1.65	0.167	5.41	46.24
20	1.57	0.025	1.77	0.080	7.25	57.99
200	2.30	0.024	1.98	0.110	5.77	39.30

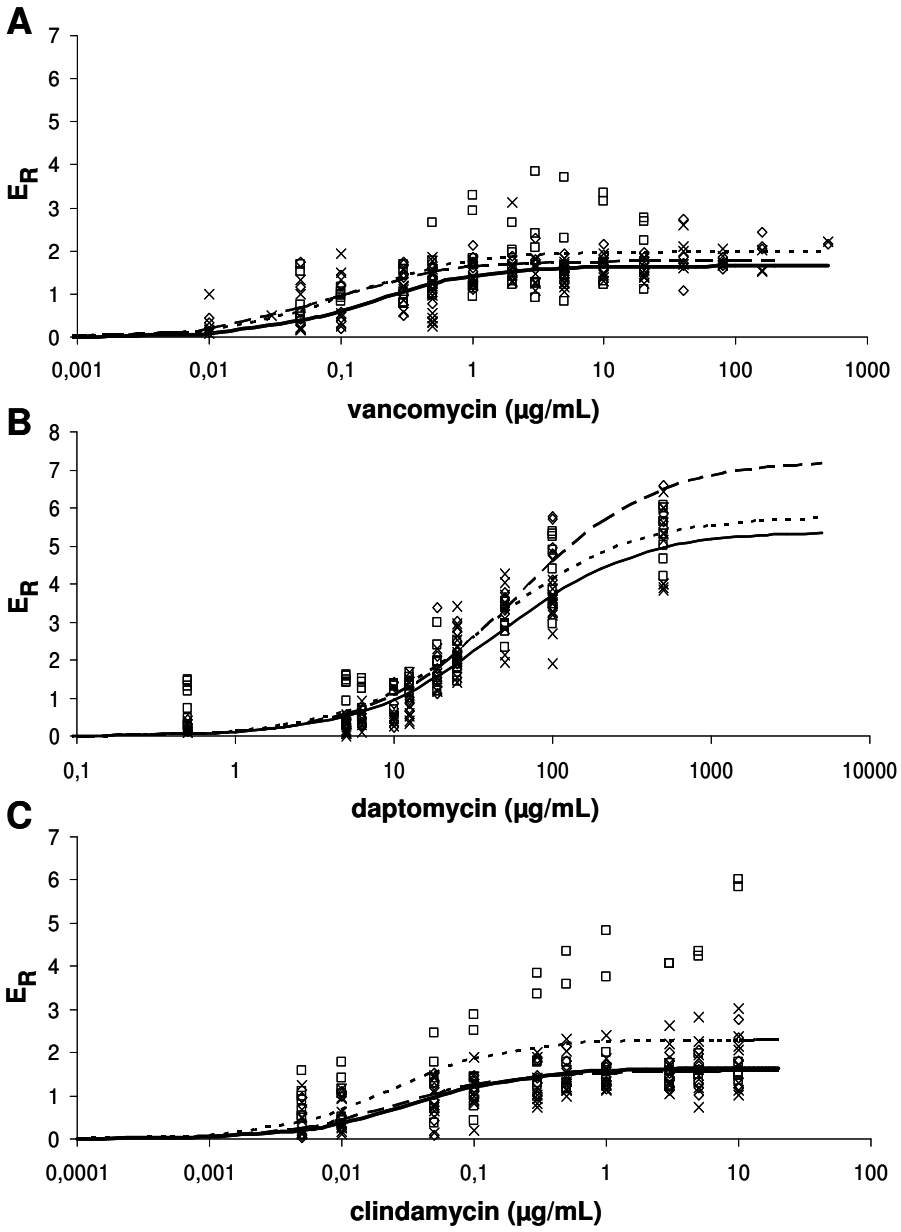

Figure 3 Effect of ES on the concentration-effect relationships of the antibiotics against exponential growing *Staphylococcus aureus*.
Bacteria were incubated with increasing concentrations of vancomycin (A), daptomycin (B) or clindamycin (C) in the presence or absence of ES (20-200 µg/mL). Results of 6-8 experiments are shown independently and as a line after processing the data using the Hill equation (x/— = no ES; ◊/— — = 20 µg of ES/mL; □/– – – = 200 µg of ES/mL).

Discussion

The main conclusion from the present study is that after maggot excretions/secretions breakdown *Staphylococcus aureus* biofilms, the released bacteria become susceptible again to antibiotics that fail to affect the viability of biofilm-associated microorganisms. The conclusion is based on the following observations. First, ES broke down established biofilms within 3 h and this effect was not negatively or positively affected by the antibiotics. In the absence of ES, samples containing vancomycin or daptomycin, antibiotics whose activity depends on their action on the bacterial cell-envelop, lacked activity against biofilms; similar findings were observed for the betalactam antibiotic flucloxacillin (unpublished observations). In contrast, clindamycin and linezolid (unpublished data) decreased the amount of biofilm, albeit they were unable to completely eradicate it in the 24 hour incubations applied here. Second, biofilm-derived bacteria became susceptible to the action of antibiotics after addition of fresh medium, as compared to their overall lack of activity against released bacteria kept in the biofilm wells. An explanation for these results is that the bacteria derived from biofilms are in a static/dormant state and are therefore not susceptible to antibiotics that solely target growing bacteria[9]. In agreement, we found no increase in bacterial numbers in the wells in which the biofilm had been exposed to ES, whereas increased numbers of *S. aureus* were observed after transferring the bacteria to fresh medium. Daptomycin showed direct activity against biofilm-derived bacteria. Third, ES did not alter the activity of the antibiotics against exponential growing and biofilm-derived bacteria. An exception was our observation that 200 µg of ES/mL enhanced the antibacterial activity of daptomycin against biofilm-derived *S. aureus* transferred to fresh medium. Although we cannot explain these results, it likely depends on the specific pharmacodynamic mechanism of this antibiotic.

In the interpretation of the current findings, the following points need be considered. First, we tested a single strain of the Gram-positive *S. aureus*. Although the strain is an ATCC reference strain, we cannot exclude that our findings are not generalisable to other *S. aureus* strains, and/or other bacterial species. However, in agreement with our result, several reports describe that daptomycin is one of the most active antibiotic in the control of biofilm-related *S. aureus* whereas clindamycin and vancomycin are less effective[23,24]. Second, the concentrations of antibiotics used in the *in vitro* biofilm assay are relatively high compared to the free, active antibiotic concentrations generally achieved in patients (vancomycin 10-40 mg/L, daptomycin 1-15/20 mg/L, clindamycin 1-20 mg/L). Therefore, at clinically relevant concentrations, the antibiotics used in this study are not expected to differ much in their activity against exponential growing *S. aureus*. However, much higher concentrations of antibiotics can be attained in wounds, through topical application, which are similar to those tested here. Third, at MIC values vancomycin or daptomycin did not affect the biofilm size, whereas at higher concentrations biofilm formation was enhanced.

However, MIC concentrations of antibiotics did not reduce the number of viable biofilm-derived bacteria whereas the higher, biofilm-enhancing concentrations did. In agreement, supra-MIC concentrations of antibiotics are reported to be effective against killing of bacteria released from biofilms, whereas sub-MIC and MIC levels were not[25]. Fourth, in contrast to the above mentioned reports[23,24], we did not observe a reduction in biofilm size when using low levels of antibiotics. The explanation for this inconsistency could be the method of quantification. We used CV staining to quantify the amount of biomass whereas many reports describe the use of redox indicators to measure the metabolic activity of the bacteria. However, reduced metabolic activity does not exclude similar or even increased biomass. In agreement, it is reported that several antibiotics, including vancomycin, reduce the redox potential of bacteria without reducing the matrix[26]. This may lead to bacterial re-growth from the remaining matrix and may even contribute to the development of resistance against the antibiotics. Clearly, more research should be done to investigate the effect of antibiotics on bacterial biofilms and the bacteria derived from these structures.

What is the clinical relevance of our findings? The failure to affect biofilms and the associated bacteria parallels the overall lack of activity of antibiotics against bacterial colonization and infection of chronic wounds where biofilm formation may be prominent[7,8,10,27]. Therefore, biofilm matrices and the associated bacteria have to be targeted simultaneously to eradicate chronic infections. Earlier we found that maggot excretions/secretions break down biofilms of *Staphylococcus aureus*[18]. Here we report that the released bacteria become exposed and susceptible to the actions of antibiotics that fail to affect biofilm-associated microorganisms. Additionally, these bacteria will be subjected to the effector mechanisms of the immune system and ingestion by maggots[28,29]. Thus, addition of maggots or maggot ES to antibiotics for the treatment of chronically colonized/infected wound surfaces may become a promising approach in inert and unresponsive chronic wounds. Of note, antibiotics including vancomycin and clindamycin have no detrimental effects on maggot growth and survival[30]. Based on our results and other reports[23,24], daptomycin and ES combined appear particularly promising in the treatment of biofilm-related *S. aureus* wound infections. Daptomycin, in contrast to vancomycin and cationic antimicrobial peptides, kills bacteria without inducing bacterial lysis[31-33]. As chronic wounds often are marked by a prolonged and dysregulated inflammatory responses[13,34-36], decreased bacterial lysis may reduce excessive pro-inflammatory responses to bacterial products by immune cells thereby contributing to the healing process[32].

In conclusion, maggot therapy and antibiotics could be used together to combat chronically colonized inert wounds. This would ensure complete breakdown of the biofilms, thereby preventing bacterial re-growth from the remaining matrix, and prompt antibiotic action against the bacteria released from the biofilms. In this respect, it should be realized that some current treatment modalities, where maggots apparently are used as replacement instead of adjunct to antibiotics, often overestimate bacterial killing by ES when applied in

therapeutically relevant amounts[18]. The combination of antibiotics and maggots will most likely lead to faster healing as maggots, besides affecting biofilm-associated microorganisms, affect other processes in wounds as well[19,37,38].

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CHAPTER FOUR

Maggot secretions enhance plasminogen activator-induced fibrinolysis by cleavage of plasminogen

Mariena J.A. van der Plas[1,2], Anders S. Andersen[3,4,5], Sheresma Nazir[1], Paul J. Hensbergen[6], Nico H. van Tilburg[7], Peter R. Oestergaard[4], Floor Dohmen[1], Jaap T. van Dissel[1], Rogier M. Bertina[7,a] and Peter H. Nibbering[1,a]

[1] Department of Infectious Diseases and [2] Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands
[3] ABMP, Statens Serum Institute, Copenhagen, Denmark
[4] Novozymes A/S, Bagsvaerd, Denmark
[5] Copenhagen Wound Healing Center, Bispebjerg Hospital, Copenhagen, Denmark.
[6] Department of Parasitology and [7] Department of Hematology, Leiden University Medical Center, Leiden, The Netherlands
[a] These authors contributed equally to this study

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Abstract

Maggots of the blowfly *Lucilia sericata* are used for the treatment of chronic wounds. As hemostatic processes play an important role in wound healing, this study focused on the effects of maggot secretions on coagulation and fibrinolysis.
The results showed maggot secretions to enhance the plasminogen activator (tPA and uPA)-induced formation of plasmin and subsequent fibrinolysis without affecting coagulation. Secretions themselves did not induce plasmin formation. Furthermore, we found secretions to interact with plasminogen, but not with plasminogen activators, in a dose- and time-dependent manner. Using acid-urea gels and selective protease inhibitors we found that a serine protease within the secretions was responsible for cleavage of plasminogen, leading to a derivative which is activated more easily by plasminogen activators. We conclude that a serine protease within maggot secretions enhances plasminogen activator-induced fibrinolysis by cleavage of plasminogen.

Introduction

Maggots of the green bottle blowfly *Lucilia sericata* are used for the treatment of many types of wounds including venous ulcers[1], traumatic and post-surgical wounds[2], osteomyelitis[3] and burns[4]. Although maggots are known primarily for debridement - removal of necrotic tissue and fibrin slough - of chronic wounds, they exert many additional effects. Earlier we reported maggot excretions/secretions to breakdown bacterial biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*[5]. Furthermore, maggots ingest and subsequently kill bacteria in their digestive tract[6]. In addition to antibacterial effects, we showed secretions to inhibit the pro-inflammatory responses of human neutrophils[7] and monocytes[8] without affecting the antimicrobial activities of these phagocytes. Moreover, maggot secretions skew the monocyte-macrophage differentiation away from a pro-inflammatory to a pro-angiogenic type (submitted for publication). Others reported accelerated fibroblast migration induced by maggot excretions/secretions[9,10]. However, there is an overall lack of information on the effect of maggots on coagulation and fibrinolysis.

Coagulation refers to the formation of insoluble fibrin, which stops hemorrhage and provides a provisional matrix essential for cell migration thereby aiding in the repair of damaged vessels and tissues[11,12]. In a balanced wound healing process these fibrin clots are broken down (during remodelling of the tissue) in the fibrinolytic phase; plasminogen is converted by plasminogen activators (uPA or tPA) to plasmin, which subsequently cuts the fibrin mesh by proteolytic degradation[12]. In chronic wounds, fibrin clots may be partially degraded by proteolytic enzymes derived from immune cells, like neutrophils and macrophages. These clots no longer support re-epithelialisation and granulation tissue formation and therefore have to be removed[11,13]. However, this cannot be accomplished by the wound components itself as, for instance, fibrinolysis may be impaired in chronic wounds due to enhanced levels of the fibrinolysis inhibitor PAI. These processes contribute to the formation of necrotic tissue and fibrin slough which contain trapped leucocytes and are a rich source of nutrients for bacteria. If necrotic tissue and/or fibrin slough are left unattended, it is very difficult to keep the wound free of infection, to prevent excessive inflammatory responses and to ensure closure of the wound. Therefore, debridement is essential for healing of these wounds and, as mentioned above, this can be exerted by maggots. It has been reported that after debridement has been accomplished by maggots minor bleeding may occur[14]. On the other hand, it has been reported that the excretory substances from the larvae have a potent hemostatic effect in haemorrhage[15]. Based upon the above considerations and clinical observations, the aim of this study was to investigate the effects of maggot secretions on coagulation and fibrinolysis.

Materials and methods

Preparation of maggot secretions

Sterile second- and third-instar larvae of *L. sericata* were a kind gift from BioMonde GmbH (Barsbüttel, Germany). Maggot secretions were collected as described[8]. Prior to use, sterile preparations of secretions were pooled and centrifuged at 1,300xg for 5 min at 4°C to remove particulate material. Subsequently, the protein concentration of these pools was determined using the Pierce BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA) according to manufacturer's instructions. In each assay, at least 3 different pools were used.

Coagulation assays

Clot formation was measured after incubating 10 µL of secretions (final concentration 50 µg/mL) or H_2O as a control with 90 µL of citrated plasma for 10 and 30 min at room temperature (RT). To initiate the intrinsic pathway of coagulation, 100 µL of APTT reagent (Kordia Life Sciences, Leiden, The Netherlands) and 100 µL of 25 mM Ca^{2+} were added (APTT-test). The extrinsic pathway was started by adding 200 µL of Thromborel S (Dade Behring BV, Leusden, The Netherlands) to the secretions-plasma mixture (PT test) while the Thrombin time (TT-test) was measured after adding 25 µL of Thrombin (100 U/mL; Enzyme Research Laboratories Inc, South Bend, IN, USA). The time needed for clot formation was measured at 37°C.

Clot lysis assay

Clot lysis was measured by a turbidimetric method using 96-wells microlon plates (Greiner Bio-One, Alphen aan de Rijn, The Netherlands). Mixtures of 80 µL were made containing 75% citrated plasma, 7.5 U/mL of tissue-type plasminogen activator (tPA; kindly provided by TNO, Leiden, The Netherlands) and secretions (range 1.25-5 µg) or, as a control, H_2O and transferred to the wells. Subsequently, 20 µL of a second mixture was added consisting of a 100 fold dilution of Innovin (Dade Behring) in TEA-buffer (containing 25 mM triethanolamine, 0.05% Tween and 50 mM NaCl) supplemented with 100 mM $CaCl_2$. Next, the plate was shaken for 30 s after which the absorbance (405 nm) was measured 60 times with an interval of 10 min at 31°C using a Tecan reader. The time needed to obtain 50% lysis of the clot (X50) was calculated. Results, being the average X50 of samples measured in duplicate, were normalized by dividing them by the X50 obtained in the absence of secretions (Ratio).

Plasminogen activation

The effect of secretions on the kinetics of fibrinolysis was investigated in a system of purified proteins (tPA and plasminogen) using the chromogenic substrate for plasmin S2403.

Mixtures were made containing tPA (25-600 U/mL), secretions (0.78-100 µg/mL) or, as a control, H_2O, Glu-plasminogen (Plg; 0.25-2 U/mL = 0.33-2.68 µM) and 0.375 mM S2403 (Chromogenix, Milano, Italy) and transferred to 96-wells microlon plates (Greiner Bio-One). Next, the plates were shaken for 30 s after which the absorbance (405 nm) was measured 30 times with an interval of 20 s at 31°C using an ELISA reader. Subsequently, absorbance values were corrected for the absorbance in the absence of Plg and tPA (due to enzymatic activity present in secretions[16]) at each time interval. The resulting values were plotted against the time square and the slope of this line, which reflects the rate of plasmin production, was calculated for each sample. Next, the Δabsorbance/sec^2 was converted into rates of plasmin production (nM/sec) using purified human plasmin as a standard (Enzyme Research Laboratories, South Bend, IN, USA).

Acid-urea gel electrophoreses

Acid-urea gels (AU-page; 10%) were prepared as described[17]. Plg in TEA buffer was incubated with tPA and/or secretions for the indicated time intervals at 31°C and mixed with sample buffer (9.5 M urea in 5% acetic acid) in a 1:1 ratio. Next, samples were transferred to the slots and gels were run in 5% acetic acid for 90-120 min at 150V using reversed polarity. Thereafter, gels were stained with Coomassie Blue.

Partial characterization of the active component in secretions

To obtain information about the nature of the active component(s), secretions were incubated with 0.1-1% of SDS, 6 M urea, 50 mM DTT or, as a control, H_2O for 1 h at RT followed by overnight dialysis using a 12-14 kD tubing membrane (Visking, Medicell International Ltd, London, UK) against ultrapure H_2O at 4°C. In addition, secretions were boiled for 2 min or 2 h. Subsequently, the effects of treated secretions on fibrinolysis were tested in the clot lysis assay. Furthermore, secretions were incubated with Serine Protease Inhibitor Cocktail Set I (SPIC-1; Calbiochem, EMD Biosciences, Inc, La Jolla, Ca, USA) or 10 mM of PMSF at various time intervals at 31°C before testing in the AU-page.

Statistical analysis

Statistical analyses were performed using Graphpad Prism version 4.02. Differences between values obtained with samples incubated with secretions and control samples were analyzed with a paired t-test. $P<0.05$ was considered significant.

Results

Effect of secretions on coagulation

To investigate whether maggot secretions interfered with the formation of blood clots, their effect on the intrinsic pathway (APTT-test), the extrinsic pathway (PT-test) and fibrin formation (TT-test) were assessed. The results showed no effect of secretions (50 µg/mL) on coagulation (Table 1).

Table 1 Effect of 50 µg of secretions/mL on coagulation

Test	10 min		30 min	
	Control	Secretions	Control	Secretions
APTT	41.0 ± 1.4	44.3 ± 1.8	41.6 ± 2.3	41.3 ± 1.6
PT	25.3 ± 0.2	22.4 ± 0.3	24.0 ± 0.3	22.2 ± 0.2
TT	19.8 ± 0.2	20.5 ± 0.6	20.5 ± 0.4	20.2 ± 0.6

The results, expressed in seconds, are means ± SEM of 6 experiments.
PT, Prothrombin Time; TT, Thrombin Time.

Effect of secretions on tPA-induced fibrinolysis

To investigate whether maggot secretions affect breakdown of plasma clots, a turbidimetric method was used. The results showed that secretions dose-dependently decreased the lysis time (Table 2; Figure 1). Secretions added to the wells 15 min after the fibrin clot was formed also reduced the lysis time -although less efficient- indicating that secretions are effective against preformed clots as well (Table 3). Similar results were obtained when using uPA (30 U/mL) instead of tPA (data not shown). Importantly, the addition of a plasminogen activator was essential as secretions themselves did not induce clot lysis. When the experiments were repeated with plasma deficient in α2-antiplasmin or TAFI, similar effects were obtained as in normal plasma indicating that the accelerating effect of secretions on fibrinolysis was not due to inhibition/inactivation of these fibrinolysis inhibitors. Two min of boiling was sufficient to abrogate the profibrinolytic effect of secretions, indicating that the active component is likely a protein (n = 3). Treatment of secretions with SDS or urea abrogated their activity (n = 3) indicating that the tertiary structure of the protein(s) is essential for its biological activity. As DTT had no effect on the activity of secretions (n = 3) it seems unlikely that the active component contains disulfide bonds.

Table 2 Effect of secretions on the lysis time of plasma clots

Secretions (µg/mL)	X50 (min)	Ratio
0	207 ± 2	1.00
12.5	195 ± 5	0.94 ± 0.03
25	181 ± 3	0.87 ± 0.02**
50	165 ± 3	0.79 ± 0.02**

Results, expressed as the time needed to obtain 50% lysis of the clot (X50) and as Ratio (X50-secretions/X50-control), are means ± SEM of 7-8 experiments. **Values are significantly ($p<0.005$) different compared to the control.

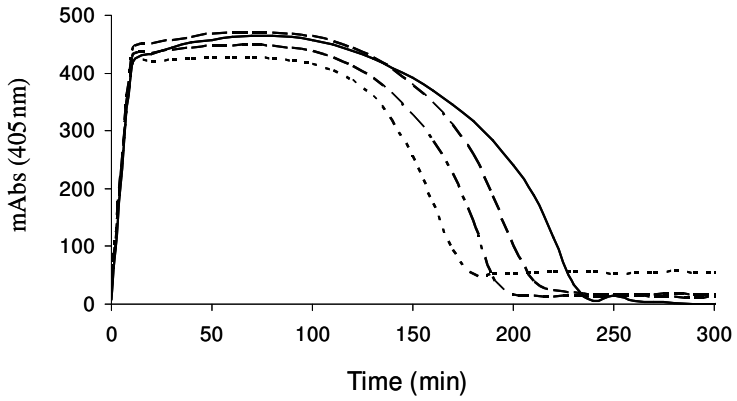

Figure 1 Representative example of the effect of maggot secretions on the tPA-induced lysis of plasma clots. No secretions —; 12.5 µg of secretions/mL — —; 25 µg of secretions/mL – —; 50 µg of secretions/mL – – –.

Table 3 Effect of secretions on the lysis time of established plasma clots

Secretions (µg/mL)	Ratio
0	1.00
25	0.96 ± 0.04
50	0.92 ± 0.03*
100	0.86 ± 0.06*
200	0.81 ± 0.04*
250	0.73 ± 0.02*

Results, expressed as Ratio (X50-secretions/X50-control), are means ± SEM of 6-8 experiments. *Values are significantly ($p<0.05$) different compared to the control.

Stimulation of tPA-induced plasmin formation by secretions

As the presence of a plasminogen activator was essential for secretions-stimulated fibrinolysis, we further studied the effect of secretions on the tPA-induced plasminogen activation. The results showed secretions to enhance the rate of plasmin formation in the presence of 0.33 µM of Plg and 50 U/mL of tPA but not in the absence of tPA (Figure 2A). Maximum stimulation was observed for 12.5 µg of secretions/mL; this concentration was independent of the tPA concentration used (data not shown). In contrast, maximum stimulation was obtained at higher secretions concentrations when the Plg concentration was increased (Figure 2B). These data indicate that secretions interact with Plg but not with tPA.

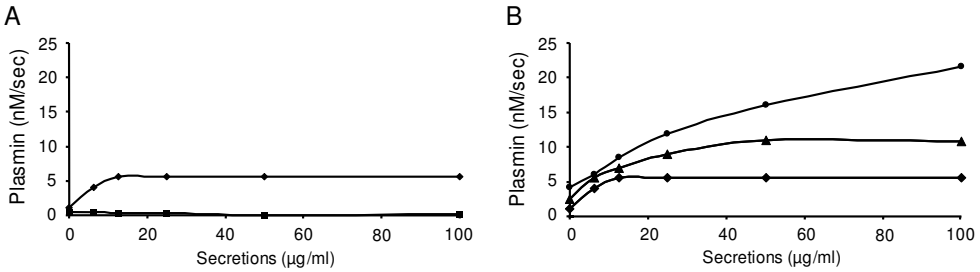

Figure 2 Effect of secretions on the tPA induced conversion of Glu-plasminogen.
A) The effect of secretions (1.56-100 µg/mL) on plasmin formation from 0.33 µM of Plg and 50 U/mL of tPA (diamonds) or no tPA (squares). B) The effect of secretions (1.56-100 µg/mL) on plasmin formation from 0.33 µM (diamonds), 0.67 µM (triangles) and 1.34 µM (circles) of Plg/mL in the presence of 50 U/mL of tPA. The shown results are of representative examples out of 3-6 experiments.

To investigate this interaction in more detail, secretions and plasminogen (0.33 µM) were incubated for various time intervals before addition of tPA (50 U/mL) and the plasmin substrate. The results showed that plasmin formation was dependent on the pre-incubation time (Figure 3A), indicating that enzymes in the secretions are responsible for the observed stimulatory effect. Plg incubated with secretions (12.5-100 µg/mL) for 24 h was no longer activated by tPA although lower concentrations of secretions were still able to stimulate Plg activation. Incubating tPA with secretions before addition of Plg and the plasmin substrate had virtually no effect on plasmin formation (Figure 3B).

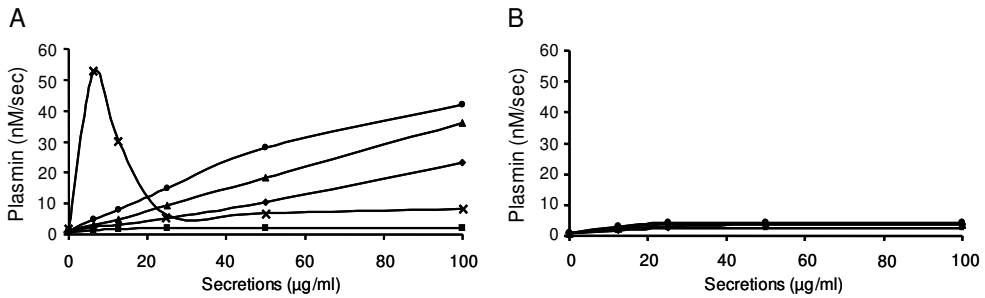

Figure 3 Effect of pre-incubation of Glu-plasminogen or tPA with secretions on plasmin formation. 0.33 µM Glu-Plg (A) or 50 U/mL of tPA (B) were pre-incubated with secretions for various time intervals before measuring plasmin formation. Pre-incubation time: 0 min = squares; 20 min = diamonds; 40 min = triangles; 60 min = circles; 24 h = cross. The results are of representative examples out of 3-6 experiments.

Effect of secretions on plasminogen

As secretions enhance tPA-induced plasmin formation by interacting with Plg, we considered the possibility that secretions promote the proteolytic conversion of Plg into (a) derivative(s) with a higher affinity for plasminogen activators. The results showed secretions to cleave plasminogen in a dose- (Figure 4A) and time-dependent (Figure 4B) manner eventually leading to complete fragmentation. Incubating secretions with 10 mM of PMSF or SPIC-1 abrogated the observed cleavage of Plg (Figure 4C) indicating that a serine protease within secretions was responsible for these effects. However, formation of Lys-plasminogen, a known derivative of Glu-plasminogen[18], and/or plasmin was not observed when incubating Plg with secretions whereas incubation of Plg with tPA resulted in the formation of Lys-plasminogen and/or plasmin (Figure 4D).

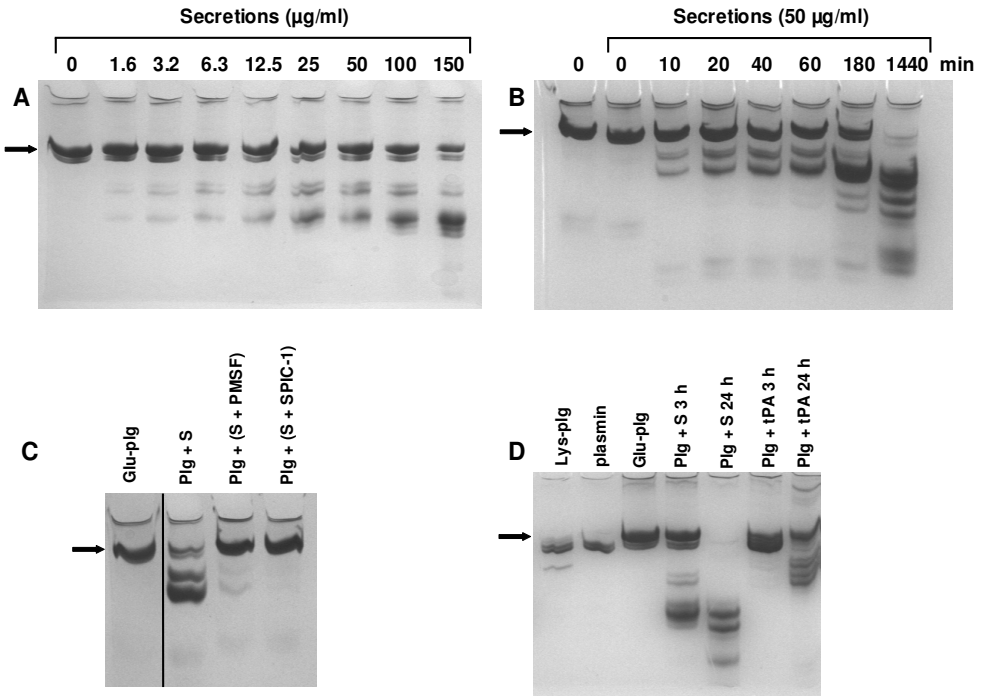

Figure 4 Effect of secretions (S) on cleavage of Glu-plasminogen visualized with AU-page. Plg was incubated with A) various amounts of secretions for 1 h, or B) with 50 µg/mL of secretions for various time intervals. C) Plg was incubated for 1 h with a mixture of 50 µg of secretions/mL pre-incubated for 24 h with the serine protease inhibitors PMSF or SPIC-1. D) The formation of Lys-plg/plasmin by secretions or tPA for 3 h and 24 h. Arrow: Glu-plasminogen.

Discussion

The main conclusion from the present study is that a serine protease present in the secretions of maggots enhances plasminogen activator-induced fibrinolysis by cleavage of plasminogen. This conclusion is based on the following observations. First, the tPA (and uPA) induced lysis of fibrin clots was enhanced by secretions when added either before or after the formation of clots from plasma; secretions could not induce lysis in the absence of these activators. Secretions exerted similar effects on clots formed with TAFI or α2-antiplasmin deficient plasma indicating that the observed enhanced fibrinolysis was not the result of inactivation of these inhibitors. Second, using a system of purified proteins, we observed secretions to enhance the tPA-induced plasminogen activation in a dose-dependent manner. Pre-incubating Plg with secretions further enhanced this process whereas pre-incubation of tPA with secretions did not lead to increased plasmin formation. Third, secretions were inactive after treatment with SDS and urea as well as after boiling

indicating that the active component is a protein with a tertiary structure essential for its biological activity. We hypothesized that protease activity in secretions[19] was responsible for cleavage of plasminogen into a derivative that is activated more easily by plasminogen activators. Using AU-page we found a serine protease within secretions to cleave Glu-plg in a dose- and time-dependent manner, although no formation of the Lys-plg[18] and/or plasmin was observed. Moreover, using the plasmin substrate assay we found secretions to enhance the tPA-induced plasmin formation from purified Lys-plg as well (data not shown). Therefore, digestion of Plg by secretions probably leads to formation of a small derivative like miniplasminogen (kringle 5 attached to the protease part)[20], which still can be activated to plasmin by plasminogen activators. In summary, a serine protease within secretions enhances the plasminogen activator-induced fibrinolysis by cleavage of Plg though the cleavage site itself is unknown and should be further investigated.

The second conclusion from this study is that maggot secretions have no effect on coagulation, at concentrations that clearly affect fibrinolysis. Therefore, the observations by Weil *et al* that the excretory substances from the larvae have a potent hemostatic effect in haemorrhage[15] may be due to other, yet unknown, causes. However, no other reports mention effects of maggots on haemorrhage as observed by Weil. In contrast, it has been reported that bleeding may occur after debridement of wounds by maggots. A possible explanation could be that the clot is broken down before the underlying tissue has healed. However, in none of the patients treated with the contained form of maggot therapy (biobags) has bleeding been observed[21]. It is unclear whether bleeding is the result of crawling of maggots[22] or of the amount of lysing components as it is likely that a large part of the maggot products stick to the biobags leading to less active molecules in the wounds.

What could be the clinical relevance of our findings? In a balanced wound healing process proteases are involved in autolytic/enzymatic degradation of the clot/provisional matrix which is essential for remodelling and repair of the tissue[23]. However, proteases, such as elastase and matrix metalloproteases (MMPs), in chronic wounds not only partially degrade clots and extracellular matrix but, due to excessive proteolytic enzyme production by immune cells[11,13,24], also damage surrounding healthy tissue. The clots no longer support re-epithelialisation and granulation tissue formation and therefore have to be removed. However, elevated levels of pro-inflammatory mediators, like TNF-α and C5a, in chronic wounds may lead to enhanced production of the fibrinolysis inhibitor PAI-1[25,26] as is reported for obese and diabetic patients[27,28]. PAI-1 binds to and inactivates uPA and tPA which results in impaired lysis of clots and fibrin cuffs[27,29]. Additionally, enhanced levels of methylglyoxal found in diabetic patients result in decreased activation of plasminogen[30]. Clearly, failure in the removal of impaired clots may promote formation of ulcers and/or (more) necrotic tissue and/or fibrin slough, which facilitates bacterial colonization and infection and consequently pro-inflammatory responses. In contrast to many ‘conventional’ therapies, maggots actively induce healing of chronic wounds. Maggots not only inhibit the

production of pro-inflammatory mediators by leucocytes[7,8] but also debride the wounds of necrotic tissue and fibrin slough. Debridement is often explained to result from enzymatic activity of the excretions/secretions of the maggots involving serine proteases, peptidases, and lipases[31-33]. However, our results showed that secretions do not degrade plasma clots in the absence of plasminogen activators. This is in contrast with the results of Chambers *et al*[16] who reported that maggot ES induced lysis of a fibrin matrix and various clot/matrix components suspended in Tris-HCL (pH 8), such as fibronectin, laminin and acid-solubilized collagen types I and III. This discrepancy was not the result of differences in collecting the maggot products (data not shown) but likely the result of differences in the composition of the clots. Chambers *et al* formed 'clots' containing fibrin only, whereas in our studies clots were formed using plasma which contains a large variety of other proteins that can be incorporated in the clot. Hence, the composition of the clot may be an important factor for the activity of the enzymes within secretions. In agreement, clots/matrices in chronic wounds obtain an altered composition and structure compared to those in acute wounds as some of their components have been degraded by proteases[13,34,35]. This altered structure/ composition may result in direct debridement of wounds by enzymes in secretions due to easier accessibility to the different clot/matrix components.

It should be noted that the relation between chronic wounds and the levels of tPA, uPA and PAI-1 are unclear. Classically, uPA is associated with cell-mediated activation of plasminogen in tissue whereas tPA is related to lysis of fibrin clots in the circulation[36]. However, both uPA and tPA are present in chronic ulcers. Elevated levels/activity of uPA, tPA, and PAI-1 have been reported, as well as no differences in and/or decreased activity[24,37,38]. Clearly, the localisation, activity and subsequent role of tPA, uPA and PAI-1 in chronic ulcers remains unclear and should be investigated in more detail. However, the observation that serine protease inhibitors, like PAI-1, prevent fibroblast migration into fibrin clots[39], as is seen in chronic wounds, suggests the absence of active molecules involved in plasmin formation. In agreement, impaired wound healing observed in uPA/tPA double-deficient and plasminogen-deficient mice results from the diminished ability of wound edge cells to migrate through the provisional and/or extracellular matrix[40,41].

In summary, a serine protease within maggot secretions enhances the plasminogen activator-induced fibrinolysis by cleavage of plasminogen making suboptimal levels of plasminogen activators likely sufficient to obtain lysis of clots as well as fibrin cuffs below the wound surface. These effects of secretions may explain their ability to effectively debride wounds which may be further enhanced by direct enzymatic activity of the secretions. In addition, maggot secretions enhance cAMP levels in leucocytes[7,8] which leads to reduced production of pro-inflammatory cytokines and PAI-1 further enhancing fibrinolysis. Taken together, these actions of secretions may prevent ongoing inflammation and tissue destruction in chronic wounds and hence contribute to the beneficial effects of maggot treatment.

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CHAPTER FIVE

Maggot excretions/secretions inhibit multiple neutrophil pro-inflammatory responses

Mariena J.A. van der Plas[1,2], Anne M. van der Does[1], Mara Baldry[1], Heleen C.M. Dogterom-Ballering[1], Co van Gulpen[1], Jaap T. van Dissel[1], Peter H. Nibbering[1,a] and Gerrolt N. Jukema[2,a]

[1] Department of Infectious Diseases and [2] Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands

[a] These authors contributed equally to this study

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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).

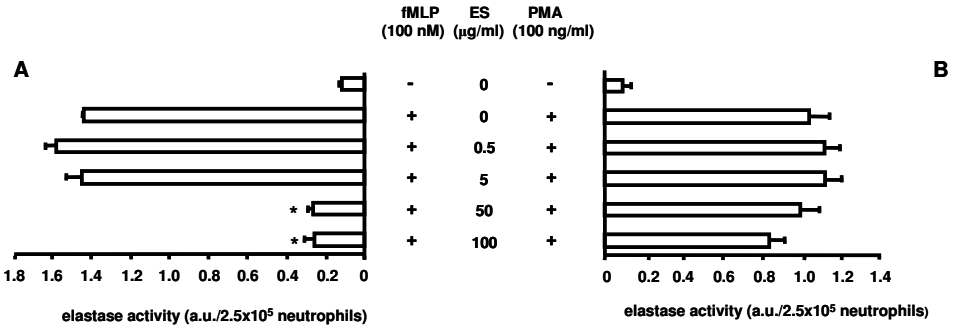

Figure 1 Effect of maggot excretions/secretions on elastase release by neutrophils in response to fMLP (A) and PMA (B). Briefly, neutrophils were incubated with fMLP (100 nM) or PMA (100 ng/mL) together with ES (range 0.5-100 µg/mL) or no stimulus. After incubation for 30 min at 37°C, the reaction was 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.

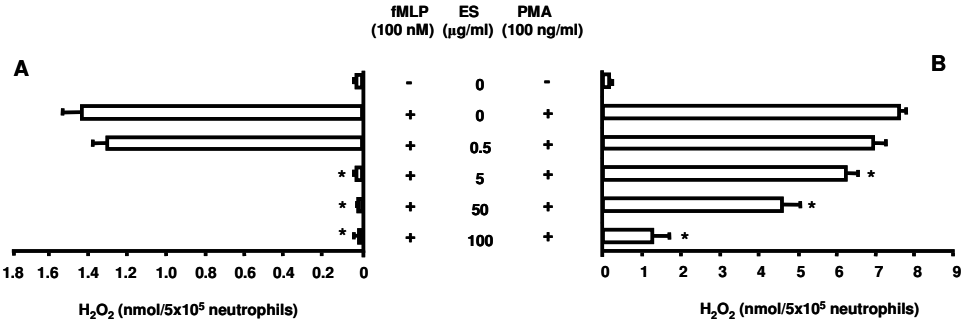

Figure 2 Effect of maggot excretions/secretions on the H_2O_2 production by neutrophils in response to fMLP (A) and PMA (B). The production of H_2O_2 by neutrophils upon stimulation with fMLP (100 nM) or PMA (100 ng/mL) in the presence of ES (range 0.5-100 µg/mL) or no stimulus was determined using the 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.

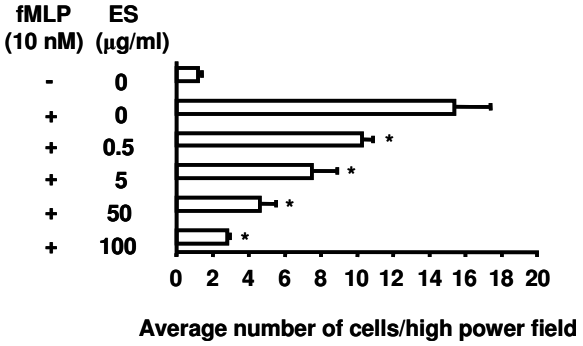

Figure 3 Effect of maggot excretions/secretions on the fMLP-stimulated neutrophil chemotaxis. Migration of neutrophils in response to 10 nM fMLP was monitored using a Boyden microchemotaxis chamber. In short, fMLP together with ES (range 0.5-100 µg/mL) or as a control with buffer was pipetted in the 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.

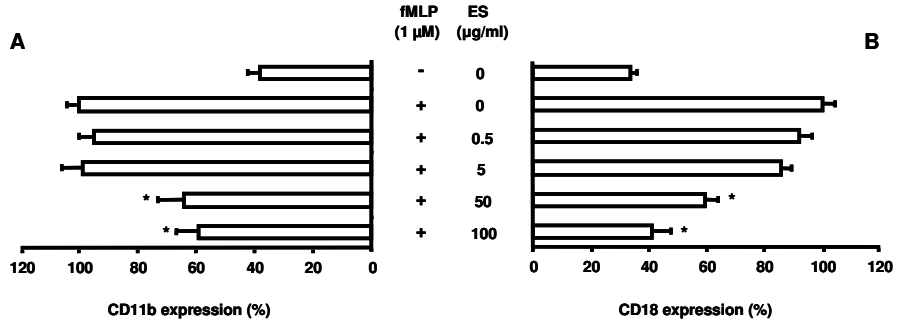

Figure 4 Effect of maggot excretions/secretions on fMLP-induced CD11b (A) and CD18 (B) expression by neutrophils. In short, neutrophils were incubated with fMLP (1 µM) together with ES (range 0.5-100 µg/mL) or as a control no ES for 1 hr at 37°C. Thereafter, cells were washed and reincubated for 30 min 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).

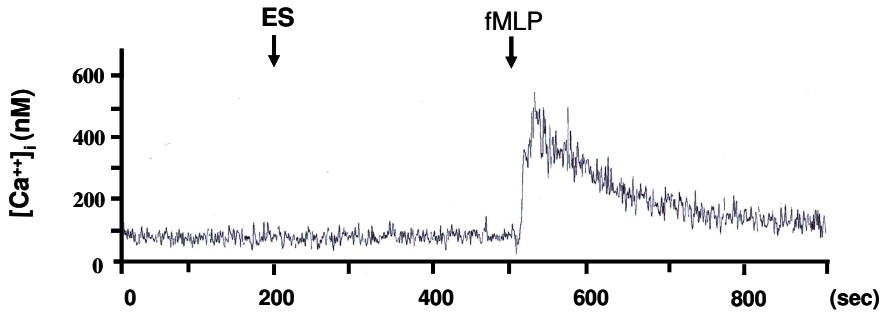

Figure 5 Effect of maggot excretions/secretions on fMLP-induced increase in the $[Ca^{++}]_i$ in neutrophils. Fura-2-loaded neutrophils were transferred to a quartz cuvette and then placed in a spectrophotometer. Thereafter, the samples were excited at 340 nm and 380 nm using an emission wavelength of 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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CHAPTER SIX

Maggot secretions suppress pro-inflammatory responses of human monocytes through elevation of cAMP

Mariena J.A. van der Plas[1,2], Mara Baldry[1], Jaap T. van Dissel[1], Gerrolt N. Jukema[3] and Peter H. Nibbering[1]

[1] Department of Infectious Diseases and [2] Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands
[3] Department of Trauma Surgery, Free University Medical Center, Amsterdam, The Netherlands

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Abstract

Aims Maggots of the blowfly *Lucilia sericata* are used for the treatment of chronic wounds. As monocytes may contribute to the excessive inflammatory responses in such wounds, this study focussed on the effects of maggot secretions on the pro-inflammatory activities of these cells.

Methods Freshly isolated monocytes were incubated with a range of secretions for 1 h and then stimulated with LPS (range 0-100 ng/mL) or LTA (range 0-5 µg/mL) for 18 h. The expression of cell surface molecules, cytokine and chemokine levels in supernatants, cell viability, chemotaxis and phagocytosis and killing of *Staphylococcus aureus* were measured.

Results Maggot secretions dose-dependently inhibited the production of the pro-inflammatory cytokines TNF-α, IL-12p40 and MIF by LPS- and LTA-stimulated monocytes while enhancing the production of the anti-inflammatory cytokine IL-10. Expression of cell surface receptors involved in pathogen recognition remained unaffected by secretions. In addition, maggot secretions altered the chemokine profile of monocytes by down-regulating MIP-1β and up-regulating MCP-1 and IL-8. Nevertheless, chemotactic responses of monocytes were inhibited by secretions. Furthermore, maggot secretions did not affect phagocytosis and intracellular killing of *S. aureus* by human monocytes. Finally, secretions induced a transient rise in the intracellular cyclic AMP concentration in monocytes and Rp-cAMPS inhibited the effects of secretions.

Conclusion Maggot secretions inhibit the pro-inflammatory responses of human monocytes through a cAMP-dependent mechanism. Regulation of the inflammatory processes by maggots contributes to their beneficial effects on chronic wounds.

Introduction

Chronic wounds are common in patients with vascular insufficiencies and underlying chronic conditions such as diabetes mellitus, as well as patients suffering from acute, extended trauma[1]. Of the patients with diabetes, up to 15% of the more than 200 million patients worldwide develop a foot ulcer at some stage, leading to over 1 million amputations every year[2]. The importance of chronic wounds in the pathway to lower limb amputation is paramount as 84% of amputations are preceded by a diabetic foot ulcer[3]. Chronic wounds and amputations in persons with diabetes often result in decreased physical, emotional and social function of patients, a reduced quality of life and major economic costs for both the patients, their families and society[4,5].

Sterile larvae -maggots- of the blowfly *Lucilia sericata* are used for the treatment of different types of wounds including diabetic foot ulcers[6-9]. The success rate of this therapy is around 68% for wounds unresponsive to conventional therapies although some characteristics (chronic limb ischaemia, wound depth, and age) may negatively influence the outcome[8]. Besides the removal of necrotized tissue and infectious microorganisms, maggots potently inhibit the pro-inflammatory responses of human neutrophils without affecting their antimicrobial activities[10]. Another prominent type of phagocyte in wounds is the monocyte. In response to chemotactic substances these cells migrate from the blood into the infected tissue to combat invading micro-organisms. In addition, monocytes regulate the inflammatory process by secreting cytokines and growth factors thereby recruiting more inflammatory cells and by antigen processing/presentation and lymphocyte activation.

In contrast to acute wound healing, chronic wounds are marked by a prolonged and dysregulated inflammatory phase. Inflammatory cells like neutrophils, monocytes and macrophages are not only present in excess numbers[11-13], they also have an enhanced production and release of pro-inflammatory cytokines, proteases and reactive oxygen species leading to growth factor inactivation and tissue destruction[14]. Therefore, inhibition of the pro-inflammatory responses of these cells could restrict their deleterious effects and thus contribute to healing processes. To obtain more insight in the mechanisms underlying the beneficial effects of medicinal maggots, this study focussed on the effects of maggot excretions and/or secretions on the pro-inflammatory activities of human monocytes.

Material and Methods

Maggots and their excretions/secretions

Sterile second- and third-instar larvae of *L. sericata* were a kind gift from BioMonde GmbH (Barsbüttel, Germany). Maggot excretions/secretions (ES) were collected as previously described[10]. Next, maggots were incubated for 1 h in H_2O to remove their excretions,

washed and then their secretions (S) were collected as described for ES. In the assays ES and S preparations were tested simultaneously, which is indicated as ES/S.

Isolation of human monocytes

PBMCs from healthy donors were isolated from buffy coats by Ficoll Amidotrizoate (ρ=1.077 g/mL) density centrifugation at 700xg for 20 min. Cells from the interphase were washed three times and monocytes were purified using anti-CD14 coated Microbeads (Miltenyi Biotec GmbH, Germany). When testing antimicrobial activities, cells in the interphase were used to avoid possible functional impairment of the monocytes by the interaction of CD14 with anti-CD14 coated microbeads. For the chemotaxis assay lymphocytes were removed from the interphase using anti-CD3 microbeads (Miltenyi) to avoid obstruction of the filters by large numbers of these cells.

Stimulation of monocytes

Approximately $1x10^6$ monocytes/mL of RPMI-1640 supplemented with 2 mM glutamax-I/glutamine, 2 mM penicillin/streptomycin and 10% inactivated foetal calf serum (standard medium) were transferred to wells of a 24-wells plate and incubated with ES/S or, as a control, H_2O for 1 h followed by stimulation with LPS (Sigma Chemical Co., St. Louis, MO, USA), LTA (Invivogen, Toulouse, France) or no stimulus. After 18-22 h incubation at 37°C and 5% CO_2, supernatants were collected and stored at -70°C.

Measurement of cytokine and chemokine levels

The cytokine and chemokine levels in the supernatants of the cell cultures were assessed using BioSource CytoSet™ (Biosource Europe, S.A., Belgium) and Bio-Plex kits (BIO-RAD, Hercules, CA, USA).

Chemotaxis

Migration of monocytes was measured as previously described[10] with the following modifications. The lower compartments contained a mixture of 25% HEPES buffer and 75% supernatants from monocyte cultures stimulated as described above. To test direct effects of ES/S on monocyte migration, 10 nM fMLP (Sigma) was added as well. In the upper compartment, 50 µL of $2x10^6$ monocytes/mL of RPMI-1640 were placed. Results are expressed as the number of cells counted in 2 μm^2 areas in 11 subsequent levels within each filter.

Phagocytosis assay

S*taphylococcus aureus* 42D were grown overnight in TSB at 37°C while shaking, then washed and resuspended ($1x10^7$/mL) in HBSS-0.1% (v/wt) gelatin. Equal volumes of this suspension and a freshly isolated or 18h (ES/S)-incubated monocyte suspension ($1x10^7$/mL

of HBSS-0.1% gelatin) were mixed and 10% AB-serum was added. Subsequently, 100 µL of this mixture were transferred to hydron-coated NUNCLON™ Surface plates (Nalge Nunc International, Rochester, NY, USA) containing ES/S or H_2O. At various intervals after incubation at 37°C while shaking, cells and bacteria were harvested in cold HBSS and centrifuged at 140xg for 6 min. Next, the number of non-cell-associated bacteria was determined microbiologically using serial dilutions which were plated onto agar plates. Phagocytosis is expressed as the percentage decrease of non-cell-associated *S. aureus*.

Intracellular killing assay

Opsonisation and intracellular killing of *S. aureus* were done as previously described[15] using hydron-coated NUNCLON™ plates. Disruption of monocytes was performed by harvesting these cells in H_2O supplemented with 0.01% (v/wt) BSA and then vortexing these suspensions for 60 s. Killing is expressed as the percentage decrease in the number of viable bacteria determined as described above.

Flow cytometry

Cells were incubated with FITC- or PE-conjugated monoclonal antibodies directed against CD11b, CD14, CD32, CD35, CD54, and CD64 (BD Pharmingen™, BD BioSciences, Erembodegem, Belgium), CD16 (EuroBioSciences GmbH, Friesoythe, Germany) and CD282 (TLR-2) and CD284 (TLR-4; Hycult Biotechnology, Uden, The Netherlands) in PBS containing 0.5% (w/v) BSA for 30 min on ice. Analyses were performed on the FACSCalibur (Becton&Dickinson, La Jolla, CA, USA) in combination with CellQuest™ Pro 4.0.2 software.

Cell viability

Monocytes were stimulated and incubated as described above and then incubated with fluorescently-labelled Annexin V (2.5 µg/mL, Sigma) and propidium iodide (1 µg/mL, Sigma) in 10 mM HEPES (pH 7.4) as previously described[16]. The mean fluorescence intensities of the cells were analyzed by flow cytometry.

Measurement of intracellular cAMP concentration

Monocytes in RPMI were incubated with a range of ES/S or no stimulus for various intervals up to 2 min. The reaction was stopped by adding lysis buffer. Next, the cAMP content of these samples was measured using the cAMP Biotrak Enzymeimmunoassay System (Amersham Biosciences, Buckinghamshire, UK). The cAMP concentrations were calculated from these values and the mean cell volume of human monocytes[17].

Inhibition of cAMP

Monocytes were pre-incubated with the protein kinase A inhibitor adenosine-3',5'-cyclic monophosphorothioate,triethyl ammonium salt (Rp-cAMPS; 1 mM; BioLog Life Science Institute, Bremen, Germany) for 45 min, followed by 1 h incubation with ES/S or H_2O and then stimulated for 18-22 h with 100 ng of LPS/mL. Thereafter, the cytokine production was measured.

Statistical analysis

Differences between the values for cells incubated with ES/S and those for cells incubated with H_2O were analysed with a Wilcoxon test using Graphpad Prism version 4.02.

Results

Effect of secretions on cytokine and chemokine production by monocytes

The results revealed that secretions decreased the LPS-induced production of the pro-inflammatory cytokines TNF-α, IL-12p40 and MIF by monocytes in a dose-dependent manner without effecting IL-1β or IL-6 (Table 1). The production of the anti-inflammatory cytokine IL-10 was increased by secretions. Furthermore, secretions inhibited the LPS-induced production of the chemokine MIP-1β by monocytes, increased MCP-1 and IL-8, but had no effect on RANTES. Secretions did not affect the base-line levels of IL-1β, IL-6, IL-10, IL-12p40, TNF-α, RANTES or MIP-1β (data not shown). In contrast, 70 µg of secretions/mL increased the production of MCP-1 by naïve monocytes from 15 (3-53) to 1049 (425-9063) pg/mL and that of IL-8 from 578 (136-1436) to 3236 (1879-5934) pg/mL while decreasing the production of MIF from 72 (19-318) pg/mL to below the detection limit (10 pg/mL) when using 35 or 70 µg of secretions/mL (n = 6-8).

To determine whether the effective components are secreted or excreted by maggots, we compared the effects of S pools to ES pools from the same maggots on the cytokine and chemokine profile of monocytes. The results showed better effects of S than of ES when using equal protein concentrations (data not shown). However, the protein concentration was 30 ± 2% lower for S than ES pools meaning that we used the products of more maggots when testing the secretions. Therefore, we tested the differences in effects when using the volume of the S pools necessary for getting for example 35 µg and used the same volume for testing the ES pools (which was 50 µg). The results showed equal effects of S and ES indicating that the active component is secreted by maggots. Therefore, we combined the results for S and ES and refer to it as secretions.

Table 1 Effect of maggot secretions on the LPS-induced production of various cytokines and chemokines by monocytes.

	Control cells		Maggot secretions (µg/mL)			
	Median (ng/mL)	Range (ng/mL)	0.35 (%)	3.5 (%)	35 (%)	70 (%)
IL-1β	0.5	0.2 -1.6	97 ± 4	81 ± 11	80 ± 10	91 ± 16
IL-6	25	13 - 40	89 ± 7	95 ± 4	92 ± 11	116 ± 15
IL-8	153	81 - 310	120 ± 7*	121 ± 9*	149 ± 15*	268 ± 63*
IL-10	0.4	0.05 - 2.1	108 ± 8	142 ± 11**	206 ± 35**	209 ± 35**
IL-12p40	0.3	0.1 - 5.4	98 ± 6	82 ± 6*	42 ± 7**	39 ± 10**
TNF-α	11	5 - 25	109 ± 14	84 ± 5*	29 ± 5*	19 ± 4*
MIF	0.08	0.04 - 0.2	85 ± 21	41 ± 14**	13 ± 5**	5 ± 3**
MCP-1	37	12 - 68	119 ± 8	134 ± 17	250 ± 33**	367 ± 66**
MIP-1β	20	1 - 155	107 ± 11	104 ± 20	23 ± 9**	17 ± 7*
RANTES	0.4	0.2 - 1.5	101 ± 3	109 ± 6	103 ± 10	74 ± 14

The results of the control cells, shown as the median value and the range, are set at 100%. The effect of secretions is expressed as a percentage relative to these values. The results are means ± SEM of 6-10 experiments. Values are significantly (*$p<0.05$ and **$p<0.005$) different from those for control monocytes stimulated with LPS.

Effect of secretions on the sensitivity of monocytes to LPS and LTA

The results revealed that the production of TNF-α by monocytes was down-regulated significantly by 35 µg of secretions/mL for all concentrations of LPS (Figure 1A). The IL-12p40 production by monocytes was dose-dependently inhibited by secretions (Figure 1B) while the production of IL-10 by monocytes was enhanced (Figure 1C). In addition, secretions (35 µg/mL) reduced the production of TNF-α (Figure 2A) and IL-12p40 (Figure 2B) by monocytes in response to LTA dose-dependently, while enhancing IL-10 (Figure 2C).

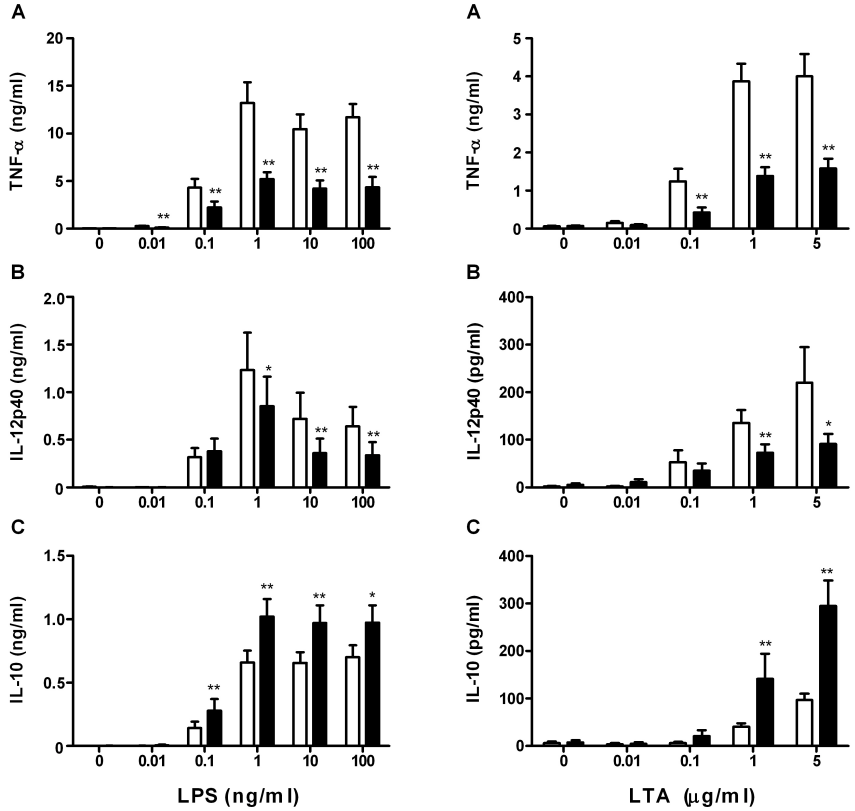

Figure 1 Effect of maggot secretions (35 µg/mL) on the production of TNF-α (A), IL-12p40 (B) and IL-10 (C) by monocytes challenged with a range of LPS. The results are means and SEM of 10-11 experiments. Values are significantly (*p<0.05 and **p< 0.005) different from those for control-incubated monocytes stimulated with LPS.

Figure 2 Effect of maggot secretions (35 µg/mL) on the production of TNF-α (A), IL-12p40 (B) and IL-10 (C) by monocytes challenged with a range of LTA. The results are means and SEM of 10 experiments. Values are significantly (*p<0.05 and **p< 0.005) different from those for control-incubated monocytes stimulated with LTA.

Effect of supernatants of secretions-treated monocytes on cell migration

Since incubation of monocytes with secretions resulted in an altered production of several chemokines, we investigated the effect of such monocyte culture supernatants on migration of monocytes. The results revealed that the chemotactic activity of monocytes towards supernatants of LPS-stimulated monocytes was abrogated when incubated in the presence of 35 µg of secretions/mL (Figure 3A). Secretions did not induce migration of naïve monocytes. Interestingly, secretions blocked migration of monocytes towards the chemotactic factor fMLP (Figure 3B). Furthermore, the chemotactic response of monocytes

towards combinations of supernatants and fMLP was decreased in the presence of secretions.

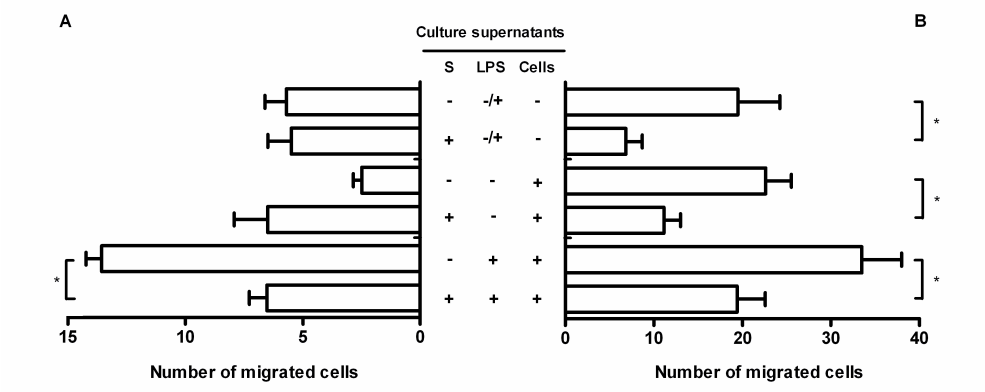

Figure 3 Effect of maggot secretions on the chemotactic activity of monocytes. Migration of monocytes in response to chemokines in cell-culture supernatants without (A) or with (B) 10 nM of fMLP was monitored using a Boyden microchemotaxis chamber. Results are means and SEM of six experiments. Each experiment was performed in quadruplicate. Values are significantly (*$p<0.05$ and **$p<0.005$) different from those for monocytes stimulated without secretions.

Effect of secretions on the phagocytosis and intracellular killing of S. aureus by monocytes

The results showed secretions (3.5 and 35 µg/mL) not to affect the phagocytosis and intracellular killing of *S. aureus* 42D by monocytes (Table 2). The antibacterial functions of monocytes incubated for 18 h with secretions were identical (data not shown).

Table 2 Effect of maggot secretions on the phagocytosis and intracellular killing of *Staphylococcus aureus* by monocytes.

Secretions (µg/mL)	Phagocytosis (%) at various intervals (min)		Intracellular killing (%) at various intervals (min)	
	30	60	30	60
0	12 ± 3	39 ± 4	38 ± 11	61 ± 7
3.5	21 ± 4	35 ± 4	44 ± 8	55 ± 10
35	19 ± 2	34 ± 3	38 ± 12	57 ± 10

Results are means ± SEM of 6 experiments.

Effect of secretions on cell surface receptors on monocytes

The results (Table 3) showed secretions (35 µg/mL) not to affect the expression of the pathogen-recognition receptors CD282, CD284 and CD14 or the Fcγ receptors CD16, CD32 and CD64, involved in the phagocytosis of opsonised bacteria, except for a reduction in the LPS-induced expression of CD32 (FcRII). Additionally, the expression of CD11b (together with CD18 complement receptor 3), involved in adhesion of monocytes to endothelial cells and phagocytosis of bacteria, was enhanced by secretions while complement receptor 1 (CD35) expression was not affected. CD54 expression was enhanced by secretions on naïve, but not LPS-stimulated, cells.

Table 3 Effect of maggot secretions on the expression of surface molecules on monocytes.

	No stimulus		LPS (100 ng/mL)	
	0	35 µg/mL	0	35 µg/mL
CD14	27 ± 3	26 ± 3	68 ± 12	60 ± 5
CD282	34 ± 2	34 ± 2	14 ± 2	13 ± 2
CD284	32 ± 2	29 ± 2	22 ± 5	18 ± 2
CD16	15 ± 4	12 ± 2	ND	ND
CD32	74 ±12	78 ± 18	273 ± 27	214 ± 19*
CD64	31 ± 14	35 ± 18	8 ± 1	8 ± 1
CD35	31 ± 4	28 ± 3	29 ± 3	28 ± 3
CD11b	243 ± 24	399 ± 52**	74 ± 14	122 ± 21**
CD54	386 ± 35	517 ± 54**	1365 ± 71	1293 ± 60

The results, expressed as the mean fluorescence intensity (MFI), are means ± SEM of 6-10 experiments. Values are significantly (*$p<0.05$ and **$p<0.005$) different from those for control-incubated monocytes. ND: not detectable.

Effect of secretions on the viability of monocytes

The results showed that secretions dose-dependently enhanced the percentage of viable monocytes (Table 4). Moreover, the LPS-induced increase in monocyte-survival was further enhanced by secretions.

Table 4 Effect of maggot secretions on the viability of monocytes

LPS (100 ng/mL)	Secretions (µg/mL)	Survival (%)	
-	0	52 ± 6	
-	0.35	54 ± 7	
-	3.5	58 ± 5*	
-	35	74 ± 4*	
-	70	80 ± 2*	
+	0	69 ± 4*	**
+	35	80 ± 3*	

The results, expressed as the percentage viable cells, are means ± SEM of 6-10 experiments. Values are significantly (*p<0.05 and **p<0.005) different from those for control-incubated monocytes.

Effect of secretions on the intracellular cAMP concentration

Analysis of the results revealed the peak cAMP concentration to be reached 15 s after the addition of secretions (35 µg/mL) and to return gradually to basal values at 120 s (data not shown). Based on these results, the 15 s interval was chosen to determine the dose-effect relation for secretions. The results revealed 3.5 µg of secretions/mL to significantly enhance the cAMP concentrations with a maximum increase up to 1.9-fold over basal level after exposure to 70 µg of secretions/mL (Table 5). In agreement, Rp-cAMPS (1 mM) significantly attenuated the inhibitory effect of secretions (35 µg/mL) on the LPS-stimulated production of TNF-α from 71 ± 5% to 41 ± 12% (n = 9; p<0.005) and of IL-12p40 from 71 ± 6% to 32 ± 14% (p<0.005), whereas it blocked (p<0.05) the increase in IL-10 production by LPS-stimulated monocytes completely.

Table 5 Effect of maggot secretions on the cAMP concentration in monocytes

Secretions (µg/mL)	cAMP (µM)
0	0.91 ± 0.07
0.35	0.97 ± 0.08
3.5	1.09 ± 0.10*
35	1.33 ± 0.13*
70	1.70 ± 0.17*

Results are means ± SEM of 10 experiments. Values are significantly (*p<0.05) different from those for control-incubated monocytes.

Discussion

The main conclusion from the present study is that maggot secretions suppress the pro-inflammatory responses of monocytes without affecting their antimicrobial activities. This conclusion is based on the following observations. First, secretions reduced the production of the pro-inflammatory cytokines IL-12p40, TNF-α and MIF by LPS-stimulated monocytes whereas the production of anti-inflammatory cytokine IL-10 was enhanced. Addition of secretions to monocytes that had already been exposed to LPS resulted in similar effects on the cytokine profile, indicating that secretions can interfere with an ongoing inflammatory reaction (data not shown). The anti-inflammatory actions of secretions are not limited to modulation of the TLR-4 pathway as secretions exerted similar effects on cells stimulated with a TLR-2 ligand. Furthermore, secretions inhibited the LPS-induced production of TNF-α and IL-12p40 by cells in whole blood (unpublished observations). However, the production of the anti-inflammatory cytokine IL-10 by blood cells was not affected by secretions suggesting that the secretions-induced increase in IL-10 production by purified monocytes may be counteracted by cellular/molecular components of whole blood. The suggestion that maggots produce IL-10[18] was withdrawn by the authors (personal communication with dr K.Y. Mumcuoglu, Dept of Parasitology, Hebrew University-Hadassah Medical School, Jerusalem, Israel). Second, secretions decreased the chemotactic response of monocytes towards fMLP as well as to the chemotactic factors in supernatants of (LPS-stimulated) monocyte cultures. These results are in agreement with our earlier finding that ES reduced the migration of human neutrophils towards fMLP[10]. The secretions induced production of chemotactic factor MCP-1 and decreased production of migration inhibitor MIF by monocytes did not increase migration indicating that secretions inhibited migration independent of the levels of these chemokines; participation of MIP-1β inhibition cannot be excluded. The effect of secretions-induced increased levels of IL-8 and CD11b are not tested within our experimental set up. The increased expression of CD54 and CD11b on naïve monocytes is unlikely to influence chemotaxis as monocytes are triggered when migrating into a wound. Third, secretions did not affect the phagocytosis and intracellular killing of *S. aureus* by freshly isolated monocytes and by 18 h cultured monocytes. This is in agreement with our earlier findings that ES had no effect on the phagocytosis and intracellular killing of *Candida albicans* by neutrophils[10]. Additionally, 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[19,20]. An important implication of the above observations that secretions interfered in a similar fashion with activation of both the TLR-2 and TLR-4 pathways is that the reported differences in effects of maggots on survival of gram-positive and gram-negative bacteria[21] are likely the result of antibacterial activity[1] and not of differential modulation of immune cell responses.

The second conclusion pertains to the mechanisms by which secretions exert their effects on monocytes. Our results showed that the effects of secretions on the cytokine/chemokine profiles of LPS- and LTA-stimulated monocytes were not caused by an altered sensitivity to these stimuli. In agreement, secretions had no effect on the expression of surface molecules involved in the recognition of the bacterial products by (LPS-stimulated) monocytes, suggesting that secretions exert their effects either downstream of these receptors or on other, yet identified (intracellular) binding partners and targets. Based on our earlier finding that ES increased the intracellular cAMP levels in neutrophils[10], we presumed a similar mechanism by monocytes and indeed found that the cAMP concentrations were enhanced dose- and time-dependently by secretions. Pre-treating monocytes with Rp-cAMPS, an inhibitor of cAMP-dependent PKA-activation, attenuated the effects of secretions on LPS-stimulated cytokine production indicating that maggots exert their effects on monocytes through a cAMP-dependent mechanism. In agreement, others reported that activation of cAMP pathways is associated with reduced production of pro-inflammatory cytokines including TNF-α, IL-12 and MIP-1β, without affecting IL-1β production, while enhancing the production of IL-10[22,23]. Furthermore, cAMP-elevation is connected to decreased migration[24,25] whereas phagocytosis by freshly isolated monocytes remains unaffected[26]. However, elevation of cAMP is also associated with a moderate reduction in phagocytosis by incubated/stimulated monocytes and macrophages[26,27] which seems to be in contrast with our data. This discrepancy can be explained by our observation that secretions enhanced the viability of monocytes; although the total phagocytosis of bacteria remained the same, the amount of phagocytosis per viable monocyte decreased. As secretions did not decrease the expression of FcR, CR1 or CD11b (part of CR3), the reduction in phagocytosis per cell may be explained by interference of signalling pathways down-stream of receptor activation[27]. Of note, cAMP is known to inhibit apoptosis in several cell types[28-30].

What could be the clinical relevance of the present findings? Although pro-inflammatory responses are essential for healing of acute wounds, they can be detrimental in chronic wounds where inflammation persists. Some histological data exists that parts of chronic wounds seem to be stuck in different phases of healing with loss of synchronicity that leads to rapid healing[12]. Some part ready for epidermal resurfacing and fibroblast proliferation could be damaged by another part that is still in the inflammatory phase[31]. It has been reported that chronic leg ulcers are associated with elevated expression of pro-inflammatory cytokines, like TNF-α and MIF, compared to acute wounds[32-34]. These cytokines enhance the production and release of a large variety of other pro-inflammatory cytokines[35,36] as well as the synthesis of several matrix metalloproteinases and serine proteases[14,36,37]. When produced in excess these pro-inflammatory responses may cause deleterious extracellular matrix destruction[38-40], and growth factor and protease inhibitor inactivation[37,41-43] and are responsible for the failure of wound healing. In addition, TNF-α activates phagocytes to

produce reactive oxygen intermediates[44,45] which can be toxic to cells like endothelial cells, fibroblasts and leucocytes and may further promote tissue proteolysis by potentiating the effects of several proteinases while inactivating proteinase inhibitors[46,47]. Together, pro-inflammatory responses may be responsible for maintenance of chronic wounds. Furthermore, TNF-α is also related to the formation of ulcers by enhancing the production of plasminogen activator inhibitor-1[48,49] which can lead to impaired lysis of pericapillary fibrin cuffs[50,51]. Importantly, although the mechanisms underlying the immunomodulatory effects of secretions on monocytes are not fully elucidated, the findings from the present *in vitro* study show that maggot secretions potently inhibit the pro-inflammatory activities of monocytes. Secretions decrease migration of cells to the wound and reduce the amount of pro-inflammatory cytokines of the cells located in the wound while their overall antibacterial activities are unaltered. Consequently, the release of other pro-inflammatory cytokines, reactive oxygen intermediates and proteases will diminish bringing tissue destruction to a halt and may result in an environment beneficial for healing.

The exiting beneficial effect of maggots in diabetic foot ulcers and other chronic wounds found in clinical studies[6-9] could well be explained by the phenomena described in this study. Besides direct antibacterial features of maggots observed in other studies[19,20], and our earlier observations that ES can inhibit the formation of and brake down bacterial biofilms[1], we found that the maggots seem to preserve the important anti-bacterial function of human leucocytes while protecting the fragile regenerating woundbed against inflammation and tissue destruction by the same inflammatory cells.

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CHAPTER SEVEN

Maggot secretions skew the monocyte-macrophage differentiation away from a pro-inflammatory towards a pro-angiogenic type

Mariena J.A. van der Plas[1,2], Jaap T. van Dissel[1] and Peter H. Nibbering[1]

[1] Department of Infectious Diseases and [2] Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands

Submitted

Abstract

Aims/hypothesis Maggots of the blowfly *Lucilia sericata* are used for the treatment of chronic wounds. Earlier we reported maggot secretions to inhibit pro-inflammatory responses of human monocytes. The aim of this study was to investigate the effect of maggot secretions on the differentiation of monocytes into pro-inflammatory (MØ-1) and anti-inflammatory/pro-angiogenic macrophages (MØ-2) as these cells play a central role in wound healing. *Methods* Freshly isolated monocytes were incubated with secretions and GM-CSF or M-CSF for 6 days and then stimulated with lipopolysaccharides (LPS) or lioteichoic acid (LTA) for 18 h. The expression of cell surface molecules and the levels of cytokines, chemokines and growth factors in supernatants were measured.
Results Our results showed secretions to affect monocyte-macrophage differentiation leading to MØ-1 with a partial MØ-2-like morphology but lacking CD163, which is characteristic for MØ-2. In response to LPS or LTA, secretions-differentiated MØ-1 produced less pro-inflammatory cytokines (TNF-α, IL-12p40 and MIF) than control cells. Similar results were observed for MØ-2 when stimulated with low concentrations of LPS. Furthermore, secretions dose-dependently led to MØ-1 and MØ-2 characterized by an altered chemokine production. Secretions led to MØ-2, but not MØ-1, producing enhanced levels of the growth factors bFGF and VEGF, as compared to control cells. The expression of cell-surface receptors involved in LPS/LTA was enhanced by secretions, that of CD86 and HLA-DR down-regulated, while receptors involved in phagocytosis remained largely unaffected. *Conclusions* Maggot secretions skew the differentiation of monocytes into macrophages away from a pro-inflammatory to a pro-angiogenic type.

Introduction

Foot ulcers of patients with diabetes mellitus are associated with tremendous health care related and social costs[1,2]. It has been observed that only two-thirds of foot ulcers will heal[3-5]. Healing of foot ulcers is essential, since a relatively high proportion will result in amputation, leading to further costs and patient suffering[6,7]. Sterile larvae -maggots- of the blowfly *Lucilia sericata* are used for the treatment of different types of wounds including diabetic foot ulcers[8-11]. Clinical observations indicate that besides the removal of necrotized tissue and infectious microorganisms, maggots actively promote healing of chronic wounds[8,12,13]. Earlier we reported that maggot secretions inhibited the pro-inflammatory responses of human neutrophils[14] and monocytes[15] through elevation of cyclic AMP. In response to local factors, monocytes migrate into the inflamed site where they may differentiate into macrophages which exhibit either pro-inflammatory or anti-inflammatory/pro-angiogenic functions. These divergent functions of macrophages are dependent mainly on the macrophage subset which is regulated by cytokines and growth factors present in the local micro-environment[16]. For example, monocytes incubated in the presence of granulocyte macrophage-colony stimulating factor (GM-CSF) develop in pro-inflammatory macrophages (MØ-1), i.e. fried egg-shaped macrophages displaying high IL-12 and low IL-10 production in response to lipopolysaccharides (LPS), while monocytes incubated with macrophage-colony stimulating factor (M-CSF) differentiate to anti-inflammatory/pro-angiogenic macrophages (MØ-2), characterized by a stretched, spindle-like morphology, expression of CD163, and low IL-12 and high IL-10 production in response to LPS. Pro-inflammatory macrophages, by secreting cytokines and chemokines, are responsible for recruiting and activating immune cells such as neutrophils, monocytes and macrophages involved in elimination of infectious agents[17]. In addition, these cytokines lead to the expression of co-stimulatory molecules on macrophages essential for T-cell activation. When the infection is cleared, the balance shifts form pro-inflammatory macrophages to macrophages with anti-inflammatory/pro-angiogenic cytokine and growth factor activities. These cells are involved in clearance of apoptotic cells[18,19], neovascularisation and fibroblast and epidermal cell proliferation[20]. Concurrently, these cells play a major role in matrix synthesis by secretion of basement membrane components, such as collagen[21,22].

Diabetic foot wounds are marked by a prolonged and dysregulated inflammatory phase. The balance between pro-inflammatory and anti-inflammatory macrophages is disturbed[23] resulting in an enhanced production and release of pro-inflammatory cytokines, proteases and reactive oxygen species which lead to growth factor inactivation and tissue destruction[24,25]. Therefore, inhibition of pro-inflammatory responses of these cells may restrict their deleterious effects, whereas the induction of anti-inflammatory/pro-angiogenic cytokine and growth factor activities may contribute to wound repair. Based on the above considerations, the aim of this study was to investigate the effects of maggot secretions on

the differentiation of monocytes into pro-inflammatory and anti-inflammatory/pro-angiogenic macrophages.

Materials and Methods

Maggots and maggot secretions

Sterile second- and third-instar larvae of *L. sericata* were a kind gift from BioMonde GmbH (Barsbüttel, Germany). Maggot secretions were collected as described previously[15]. Prior to use, sterile preparations of secretions 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. Subsequently, protein concentrations of the pools were determined using the Pierce BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA) according to manufacturer's instructions.

Isolation of human monocytes

Peripheral blood mononuclear cells from healthy donors were isolated from buffy coats by Ficoll Amidotrizoate (ρ=1.077 g/mL) density centrifugation at 700xg for 20 min. Cells from the interphase were washed three times with PBS (pH 7.4) and monocytes were purified using anti-CD14-coated Microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to manufacturer's instructions. Next, cells were centrifuged and resuspended in RPMI 1640 supplemented with 2 mmol/L glutamax-I/glutamine, 2 mmol/L penicillin/ streptomycin and 10% (vol./vol.) inactivated fetal calf serum (further referred to as standard medium).

Macrophages

MØ-1 and MØ-2 were obtained by culturing $3x10^5$ monocytes/mL of standard medium in 24-wells plates in the presence of respectively 5 ng of recombinant GM-CSF/mL (Biosource, Camarillo, Ca, USA) or 12.5 ng of recombinant M-CSF/mL (R&D Systems Europe Ltd., Abingdon, UK). After 6 days, macrophages were stimulated with LPS (0.01-100 ng/mL; Sigma Chemical Co., St. Louis, MO, USA), lipoteichoic acid (LTA; 0.01-5 µg/mL; Invivogen, Toulouse, France) or no stimulus (further referred to as naïve macrophages). After 18 to 20 h incubation at 37°C and 5% CO_2, supernatants were collected and stored at -70°C. To investigate the effect of secretions on the differentiation of monocytes to macrophages, secretions (range 0.35 - 70 µg/mL) were added to the wells at the start of the culture and the resulting macrophages are further referred to as secretions-differentiated macrophages.

Measurement of the levels of cytokines, chemokines and growth factors

The levels in the supernatants of the cell cultures were assessed using BioSource CytoSet™ (Biosource Europe, S.A., Belgium) and Bio-Plex kits (BIO-RAD, Hercules, CA, USA) according to manufacturer's instructions.

Flow cytometry

To verify the differentiation of monocytes into MØ-1 and MØ-2, macrophages were incubated with phycoerythrin-conjugated monoclonal antibodies (mAbs) directed against CD163 purchased from BD Pharmingen™ (BD BioSciences, Erembodegem, Belgium). Furthermore, cells were incubated with FITC- or phycoerythrin-conjugated mAbs directed against CD11b, CD14, CD32, CD35, CD54, CD64, CD86, HLA-DR and CD206 (BD Pharmingen™, BD BioSciences, Erembodegem, Belgium), CD16 (EuroBioSciences GmbH, Friesoythe, Germany), and CD282 (Toll-like receptor [TLR]-2) and CD284 (TLR-4; Hycult Biotechnology, Uden, the Netherlands for both); incubation was in PBS containing 0.5% (wt/vol.) BSA for 30 min on ice. Analyses were performed on the FACSCalibur (Becton&Dickinson, La Jolla, CA, USA) in combination with CellQuest™ Pro 4.0.2 software. Mean fluorescence intensities (MFI) of unstained samples were subtracted from the stained samples. MFI's below 6 are indicated as not detectable (≤ 2 times MFI unstained samples).

Statistical analysis

Differences between the values for cells incubated in the presence of maggot secretions and those for cells incubated with H_2O were analysed using a Wilcoxon test using Graphpad Prism version 4.02.

Results

Effect of maggot secretions on the differentiation of monocytes to macrophages

Light microscopy revealed macrophages that differentiated under the influence of GM-CSF to display a 'fried egg-like' morphology (Figure 1A) whereas the addition of secretions (35 µg/mL) led to MØ-1 (Figure 1B) that partially obtained a phenotype resembling MØ-2, i.e. elongated, spindle-like appearance as induced by M-CSF (Figure 1C). Secretions enhanced the development of this morphology by M-CSF differentiated macrophages (Figure 1D). However, secretions did not induce CD163 expression on GM-CSF-differentiated macrophages, which is a characteristic of MØ-2. In addition, secretions did not lead to an altered expression of CD163 (mean fluorescence intensity ~20) on MØ-2.

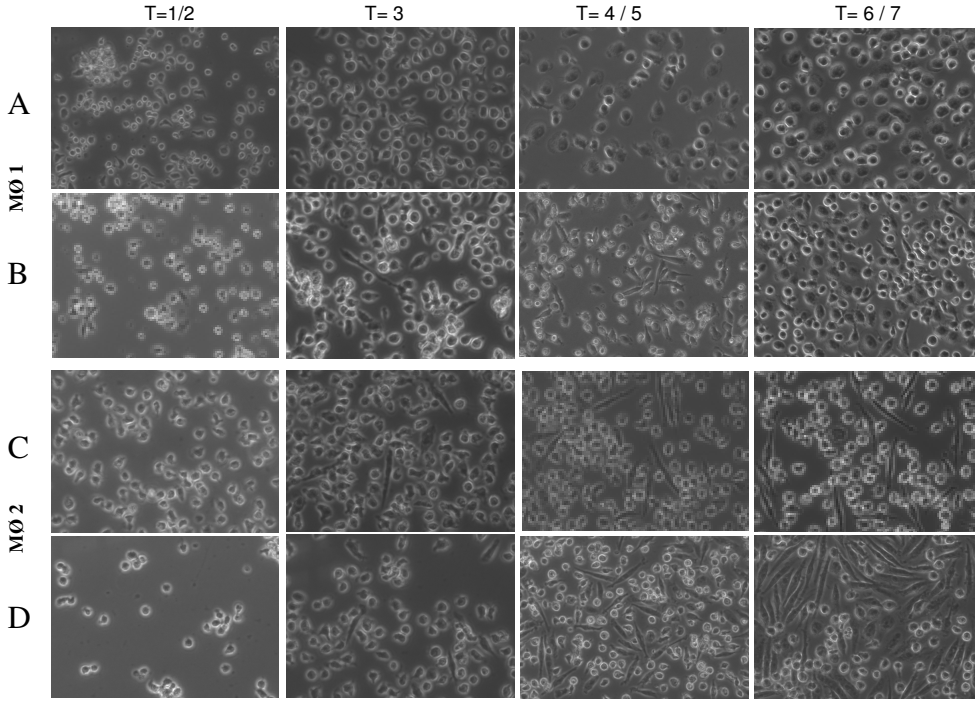

Figure 1 Light microscopy analysis of the effect of 35 µg of secretions/mL on the differentiation of monocytes to macrophages. In short, monocytes were differentiated to MØ-1 in the presence of GM-CSF (A) or in the presence of GM-CSF and secretions (B) and their morphology evaluated. Similarly, monocytes were differentiated to MØ-2 in the presence of M-CSF (C) and in the presence of M-CSF and secretions (D). Results, indicated in days, are from a representative experiment.

Further investigations showed secretions to affect macrophage differentiation resulting in MØ-1 that in response to various concentrations of LPS produced less IL-12p40 than control macrophages (Figure 2A). MØ-2 differentiated in the presence of secretions produced less IL-12p40 upon stimulation with 0.01 ng of LPS/mL, whereas 10 and 100 ng/mL led to increased IL-12p40 production compared to control MØ-2 (Figure 2B). Remarkably, the production of IL-12p40 by MØ-2 was almost 10 times higher in response to 0.01 ng of LPS/mL as compared to 100 ng of LPS/mL. The production of IL-10 by both types of macrophages differentiated in the presence of secretions did not differ from that by control macrophages (Figure 2C and 2D). Taken together, the above results indicate that maggot secretions affect the differentiation of monocytes to macrophages, but do not result in the differentiation from one type into the other.

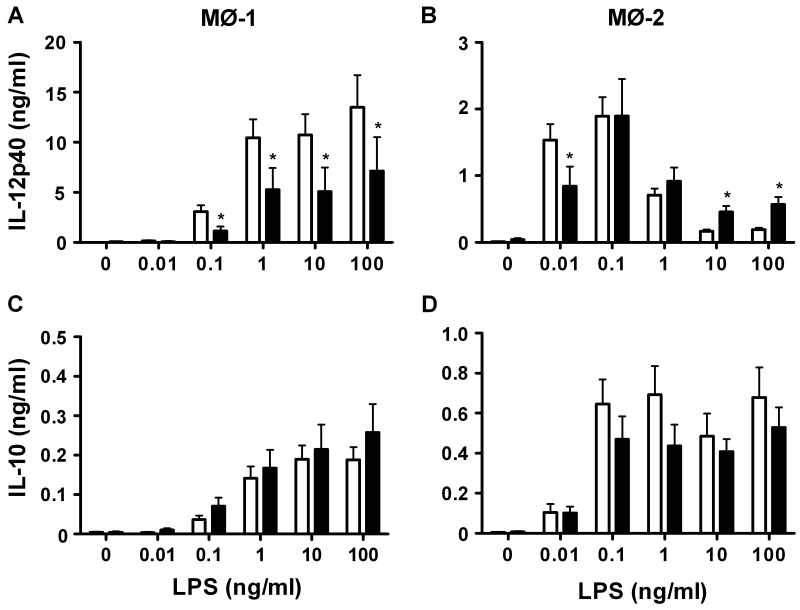

Figure 2 The production of IL-12p40 (A,B) and IL-10 (C,D) by control and secretions-differentiated MØ-1 and MØ-2 in response to a range of LPS. The results, expressed in ng/mL, are means ± SEM of 9-10 experiments. Open bars: control macrophages; filled bars: secretions-differentiated macrophages. Values are significantly (*p<0.05) different from those for control macrophages.

Cytokine production by secretions-differentiated macrophages

The results showed that maggot secretions dose-dependently gave rise to MØ-1 with a decreased production of the pro-inflammatory cytokines IL-12p40, TNF-α, and Macrophage Migration Inhibitory Factor (MIF) upon LPS stimulation, as compared to control cells (Table 1), whereas the level of IL-1β (mean 103 and range 51 - 150 pg/mL) did not differ (data not shown). In addition, the LPS-induced production of IL-6 by these cells was dose-dependently enhanced as was that of the anti-inflammatory cytokine IL-10 when using small amounts of secretions. Secretions had no effect on base-line levels of IL-12p40, TNF-α, IL-10 and IL-1β (data not shown), but 70 µg of secretions/mL led to cells with an increased ($p<0.005$) production of IL-6 from 37 (range: 0 - 125) to 128 (range: 6 - 524) pg/mL and a decreased ($p<0.05$) production of MIF from 226 (range:81 - 553) to 65 (range: 0 - 99) pg/mL.

In agreement with their effects on LPS-stimulated MØ-1, maggot secretions dose-dependently led to MØ-2 that showed a reduced production of IL-12p40 and TNF-α when stimulated with 0.01 ng of LPS/mL, as compared to control cells (Table 1). The levels of these cytokines were dose-dependently altered in the presence of secretions when stimulated with 100 ng of LPS/mL. Furthermore, secretions-differentiated MØ-2 showed a

Table 1 LPS-induced cytokine production by secretions differentiated macrophages

	Control cells				Macrophages differentiated in the presence of secretions (µg/mL)			
					0.35	3.5	35	70
	Type MØ	LPS (ng/mL)	Median (ng/mL)	Range (ng/mL)	(%)	(%)	(%)	(%)
IL-12p40	1	100	25	7.6 - 38.2	74 ± 6**	75 ± 5**	68 ± 9*	35 ± 6**
	2	0.01	1	0.2 - 3.7	72 ± 4**	70 ± 6**	33 ± 6**	22 ± 3**
	2	100	0.2	0.07 - 0.5	77 ± 4**	71 ± 7**	176 ± 22**	160 ± 28*
TNF-α	1	100	51	25.2 - 131	92 ± 5	85 ± 5*	71 ± 6**	53 ± 9**
	2	0.01	2.8	0.9 - 7.2	90 ± 4*	95 ± 7	58 ± 6**	30 ± 3**
	2	100	3.8	2.6 - 5.8	97 ± 5	100 ± 6	97 ± 8	63 ± 9*
MIF	1	100	0.2	0.07 - 0.4	60 ± 10*	42 ± 7**	19 ± 5**	24 ± 7**
	2	0.01	0.8	0.2 - 1.1	70 ± 13	49 ± 9*	19 ± 3*	15 ± 1*
	2	100	0.3	0.08 - 0.7	87 ± 23	35 ± 10**	19 ± 5**	6 ± 3**
IL-6	1	100	37	3.4 - 60.1	108 ± 8	135 ± 12**	193 ± 42**	226 ± 52*
	2	0.01	1.9	1.5 - 27.4	122 ± 9*	162 ± 14**	239 ± 28**	208 ± 26**
	2	100	0.7	0.2 - 1.4	106 ± 8	148 ± 14**	427 ± 62**	690 ± 89**
IL-10	1	100	0.1	0 - 0.6	132 ± 14*	137 ± 16*	98 ± 16	97 ± 15
	2	0.01	0.04	0.02 - 1.3	93 ± 8	114 ± 13	74 ± 12	83 ± 14
	2	100	0.7	0.2 - 1.0	102 ± 5	101 ± 8	87 ± 8	67 ± 9*

The results for the control macrophages are expressed as the median value and range, and are set at 100%. The effect of secretions is expressed as a percentage relative to the cytokine production by control cells. Results are means ± SEM of at least six experiments. Values are significantly (*$p<0.05$ and **$p<0.005$) different from those for macrophages stimulated with LPS.

reduced MIF production and an increased IL-6 production regardless of the amount of LPS used. IL-10 production was not altered by these cells when stimulated with 0.01 ng of LPS/mL, whereas 70 µg of secretions/mL led to cells with decreased IL-10 levels when stimulated with 100 ng of LPS/mL. IL-1β levels were not detectable in the supernatants of MØ-2. Secretions had no effect on base-line levels of TNF-α, IL-12p40 and IL-10 (data not shown), but 70 µg of secretions/mL led to MØ-2 displaying increased ($p<0.05$) production of IL-6 from 8 (range: 0 - 42) to 31 (range: 0 - 78) pg/mL and decreased ($p<0.005$) production of MIF from 291 (range: 165 - 567) to 33 (range: 0 - 106) pg/mL.

Chemokine production by secretions-differentiated macrophages

As influx of inflammatory cells contributes to excessive inflammation in chronic wounds, we investigated the chemokine profile of secretions-differentiated and control macrophages. The results (Table 2) showed that maggot secretions dose-dependently gave rise to naïve and LPS-stimulated MØ-1 and MØ-2 displaying increased production of Monocyte Chemotactic Protein-1 (MCP-1) and IL-8, but decreased production of Macrophage Inflammatory Protein-1β (MIP-1β). Furthermore, the production of RANTES was reduced by these cells when stimulated with LPS. RANTES was not detectable (<10 pg/mL) in the supernatants of naïve cells, but 70 µg of secretions/mL led to MØ-1 producing 20 (range: 4.5 - 661) pg/ml and to MØ-2 producing 13 (range: 3 - 24) pg/mL of this chemokine.

Growth factor production by secretions-differentiated macrophages

Since tissue synthesis and neovascularisation are essential for wound healing, we investigated the production of growth factors by macrophages differentiated in the presence of secretions. The results showed similar levels of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) in the supernatants of secretions-differentiated MØ-1 and control cells; VEGF production by naïve cells was not detectable (Table 3). Furthermore, secretions led to MØ-1 showing decreased production of platelet derived growth factor (PDGF)-BB upon LPS stimulation, whereas the production of G-CSF (median: 306 (46-1060) pg/mL) by these cells did not differ (data not shown); PDGF-BB and G-CSF were not detectable in supernatants from naïve MØ-1. Remarkably, secretions (70 µg/mL) dose-dependently led to naïve MØ-1 displaying ($p<0.005$) reduced levels of GM-CSF from 665 (range: 22 - 2505) to 138 (range: 1-1624) pg/mL and when stimulated with LPS from 556 (range: 62 - 1969) to 110 (range: 0-1216) pg/mL.

Contrastingly to MØ-1, secretions dose-dependently gave rise to naïve and LPS-stimulated MØ-2 with increased production of bFGF and VEGF. Additionally, 100 ng of LPS/mL induced a higher production of VEGF by MØ-2 than 0.01 ng of LPS/mL, while the effect of secretions was higher when these cells when stimulated with the latter amount of LPS. Furthermore, secretions gave rise to MØ-2 showing decreased production of PDGF-BB in

Table 2 LPS-induced chemokine production by secretions differentiated macrophages

	Control cells				Macrophages differentiated in the presence of secretions (µg/mL)			
	Type MØ	LPS (ng/mL)	Median (ng/mL)	Range (ng/mL)	0.35 (%)	3.5 (%)	35 (%)	70 (%)
IL-8	1	0	1.0	0.4 – 6.6	146 ± 22	467 ± 98*	2761 ± 923*	2906 ± 996*
	1	100	59	21 - 116	118 ± 14	176 ± 28*	343 ± 72*	338 ± 83*
	2	0	1.5	0.4 – 2.2	103 ± 15	142 ± 10*	552 ± 158*	716 ± 129*
	2	0.01	12	6.3 - 24	110 ± 19	149 ± 30*	250 ± 34*	378 ± 66*
	2	100	4.5	3.8 - 7.6	117 ± 10	173 ± 22*	480 ± 67*	703 ± 112*
MCP-1	1	0	0.7	0.2 - 5.8	166 ± 25*	437 ± 160*	990 ± 353*	1127 ± 471*
	1	100	8.7	3.9 - 49	135 ± 22	208 ± 50*	335 ± 256*	368 ± 85*
	2	0	31	5.9 - 40	137 ± 8*	159 ± 12*	318 ± 83*	361 ± 106*
	2	0.01	63	32 - 122	111 ± 9	161 ± 15*	189 ± 17*	175 ± 10*
	2	100	53	9.0 - 61	134 ± 35	157 ± 23*	272 ± 48**	242 ± 42**
MIP-1β	1	0	0.09	0.02 - 0.5	71 ± 5*	42 ± 7**	16 ± 4**	14 ± 4*
	1	100	25	1.2 - 338	118 ± 37	68 ± 15	28 ± 12*	6 ± 2**
	2	0	0.09	0.03 - 1.3	93 ± 4	55 ± 8**	22 ± 6**	17 ± 5**
	2	0.01	48	24 - 84	87 ± 10	51 ± 6**	6 ± 1**	4 ± 1**
	2	100	45	25 - 75	92 ± 6	61 ± 4**	9 ± 2**	4 ± 1**
RANTES	1	0	ND					
	1	100	4.6	2.1 - 15	129 ± 30	79 ± 19	28 ± 18**	21 ± 6**
	2	0	ND					
	2	0.01	0.5	0.2 - 1.5	48 ± 11**	62 ± 8**	46 ± 8**	50 ± 8*
	2	100	2.0	1.2 - 3.4	90 ± 4	78 ± 8*	47 ± 4**	34 ± 4**

The results for control macrophages are expressed as the median value and range, and are set at 100%. The effect of secretions is expressed as a percentage relative to the chemokine production by control cells. Results are means ± SEM of at least six experiments. Values are significantly (*$p<0.05$ and **$p<0.005$) different from those for macrophages stimulated with LPS. ND: not detectable.

Table 3 LPS-induced growth factor production by secretions differentiated macrophages

	Control cells				Macrophages differentiated in the presence of secretions (µg/mL)			
					0.35	3.5	35	70
	Type MØ	LPS (ng/mL)	Median (pg/mL)	Range (pg/mL)	(%)	(%)	(%)	(%)
bFGF	1	0	51	0 - 111	98 ± 6	92 ± 12	116 ± 25	150 ± 36
	1	100	58	22 - 117	97 ± 3	98 ± 4	95 ± 7	114 ± 8
	2	0	34	10 - 108	170 ± 37	199 ± 48*	305 ± 86*	372 ± 114*
	2	0.01	27	14 - 40	74 ± 19	180 ± 28*	234 ± 44*	288 ± 56**
	2	100	56	32 - 107	109 ± 4*	130 ± 13*	180 ± 30*	214 ± 42*
VEGF	1	0	ND					
	1	100	25	0 - 120	87 ± 5	94 ± 8	101 ± 16	95 ± 20
	2	0	99	18 - 195	172 ± 41*	254 ± 45**	545 ± 106**	627 ± 108**
	2	0.01	90	21 - 199	126 ± 35	825 ± 232*	1168 ± 403*	1382 ± 579**
	2	100	342	225 - 540	129 ± 10*	174 ± 18**	415 ± 57**	423 ± 103**
PDGF	1	0	ND					
	1	100	15	8 - 61	105 ± 6	98 ± 9	72 ± 5*	64 ± 9*
	2	0	20	6 - 95	115 ± 13	102 ± 8	62 ± 10*	58 ± 16*
	2	0.01	49	24 - 105	63 ± 11*	117 ± 14	54 ± 7**	40 ± 4**
	2	100	25	13 - 114	123 ± 6	112 ± 7	67 ± 3*	57 ± 5*

The results for control macrophages are expressed as the median value and range, and are set at 100%. The effect of secretions is expressed as a percentage relative to the production of growth factors by control cells. Results are means ± SEM of at least six experiments. Values are significantly (*$p<0.05$ and **$p<0.005$) different from those for macrophages stimulated with LPS. ND: not detectable

response to LPS; no PDGF-BB was detected in the supernatants of naïve MØ-2. G-CSF and GM-CSF were not detectable in the supernatants of MØ-2 (data not shown).

Expression of cell surface receptors on secretions-differentiated macrophages

To further investigate the effect of secretions on the differentiation of monocytes into macrophages, we measured the expression of cell surface molecules involved in pathogen recognition, opsono-phagocytosis, cell adhesion and T-cell activation.

The results showed that secretions (35 µg/mL) gave rise to naïve and LPS-stimulated MØ-1 and MØ-2 displaying increased expression of the pathogen-recognition receptors TLR-2 and TLR-4, as compared to control cells (Table 4). Interestingly, LPS down-regulated the expression of these two receptors on MØ-1 ($p<0.005$) but up-regulated their expression on MØ-2 ($p<0.05$). Additionally, CD14 was completely down-regulated during the differentiation of monocytes to MØ-1 but remained present on these cells when differentiated in the presence of secretions. The expression of CD14 on MØ-2 was not affected. The expression of the mannose receptor CD206 was increased on secretions differentiated MØ-1, but not on MØ-2.

Furthermore, the expression of Fcγ-receptor III, but not Fcγ-receptor II, was increased on naïve and LPS-stimulated MØ-1 and MØ-2 differentiated in the presence of secretions. Fcγ-receptor II was slightly decreased on naïve MØ-2. Secretions led to naïve MØ-1 showing enhanced expression of complement receptor 1 but no effect was seen on MØ-2 and LPS-stimulated MØ-1. In addition, the expression of CD11b (complement receptor 3, together with CD18) was enhanced by secretions differentiated MØ-1 but not affected on MØ-2. The expression of cell adhesion receptor ICAM-1 was not affected by secretions. Finally, secretions led to MØ-1 and MØ-2 with reduced expression of the co-stimulatory molecule B7.2, and to MØ-2, but not MØ-1, with decreased expression of HLA-DR.

Cytokine production by secretions-differentiated macrophages in response to LTA

The results showed that secretions gave rise to MØ-1 (Figure 3A) and MØ-2 (Figure 3B) displaying reduced levels of IL-12p40, as compared to control cells, from 0.1 ng of LTA/mL. Furthermore, secretions differentiated MØ-1 (Figure 3C), but not MØ-2 (Figure 3D), showed reduced TNF-α production regardless of the amount of LTA used. In addition, the production of IL-6 was enhanced by naïve MØ-1 (Figure 3E) and by MØ-2 for all conditions (Figure 3F) when differentiated in the presence of secretions. Secretions had no effect on the production of IL-10 by MØ-1 (Figure 3G) but led to MØ-2 showing reduced levels of this cytokine when using 1 - 5 ng of LTA/mL (Figure 3H).

Table 4 Expression of surface receptors by secretions-differentiated macrophages

		MØ-1				MØ-2					
		No stimulus		LPS (100 ng/mL)		No stimulus		LPS (0.01 ng/mL)		LPS (100 ng/mL)	
		0	35 µg/mL	0	35 µg/mL	0	35 µg/mL	0	35 µg/mL	0	35 µg/mL
CD282	TLR-2	77 ± 6	103 ± 12*	40 ± 4	62 ± 7*	54 ± 4	65 ± 6*	65 ± 6	78 ± 8*	72 ± 7	86 ± 7*
CD284	TLR-4	50 ± 4	68 ± 10*	32 ± 3	45 ± 5*	43 ± 3	51 ± 4*	55 ± 5	66 ± 6**	58 ± 5	68 ± 5**
CD14	LPS-R	ND	8 ± 2*	ND	11 ± 2*	42 ± 4	39 ± 2	41 ± 1	41 ± 3	24 ± 2	30 ± 3
CD206	MMR	45 ± 6	57 ± 7*	22 ± 2	30 ± 3*	32 ± 4	36 ± 3	27 ± 4	34 ± 5	24 ± 3	27 ± 4
CD64	FCγRI	22 ± 3	23 ± 3	12 ± 2	12 ± 2	10 ± 1	13 ± 2	11 ± 2	13 ± 3	15 ± 3	18 ± 3
CD32	FCγRII	22 ± 5	19 ± 3	19 ± 2	24 ± 4	184 ± 19	162 ± 21*	206 ± 23	195 ± 26	170 ± 18	181 ± 18
CD16	FCγRIIIA	9 ± 1	17 ± 4*	6 ± 0.4	12 ± 2*	12 ± 3	25 ± 8*	25 ± 4	37 ± 6*	11 ± 3	23 ± 9*
CD35	CR1	23 ± 3	30 ± 4*	8 ± 1	9 ± 1	15 ± 1	15 ± 1	12 ± 1	13 ± 1	8 ± 1	8 ± 1
CD11b	CR3	113 ± 23	171 ± 40*	80 ± 8	112 ± 19*	231 ± 12	235 ± 16	191 ± 14	207 ± 15	188 ± 17	206 ± 18
CD54	ICAM-1	156 ± 17	192 ± 27	374 ± 60	355 ± 66	365 ± 27	385 ± 19	920 ± 115	911 ± 134	934 ± 107	885 ± 105
CD86	B7.2	19 ± 3	14 ± 2*	105 ± 15	39 ± 5*	77 ± 7	61 ± 6**	35 ± 2	29 ± 2*	69 ± 6	42 ± 3**
HLA-DR		212 ± 36	218 ± 34	270 ± 40	249 ± 41	317 ± 42	250 ± 25*	233 ± 32	201 ± 21*	313 ± 43	239 ± 21*

Results, expressed as the mean fluorescence intensity (MFI), are means ± SEM of 6-11 experiments. Values are significantly (*$p<0.05$ and **$p<0.005$) different from those for control cells. ND: not detectable

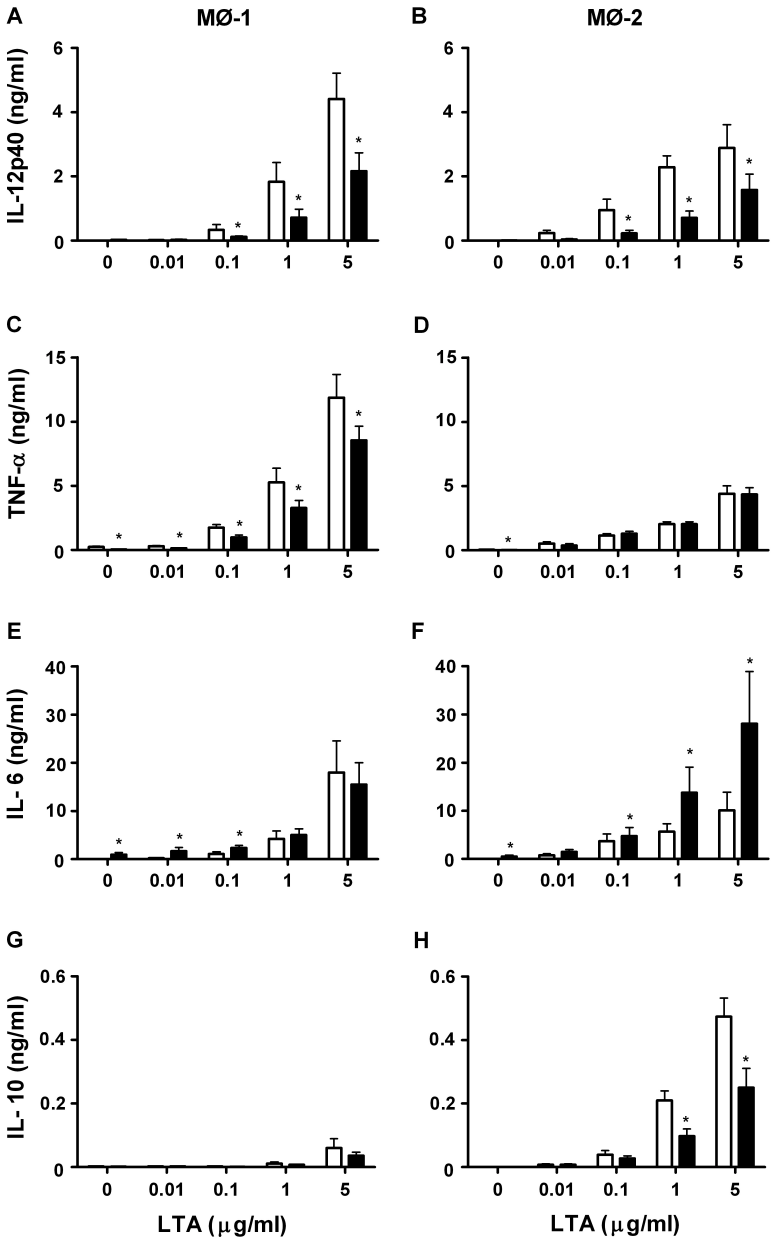

Figure 3 The production of IL-12p40 (A,B), TNF-α (C,D), IL-6 (E,F) and IL-10 (G,H)) by control and secretions-differentiated MØ-1 and MØ-2 induced by a range of LTA. The results, expressed in ng/mL, are means ± SEM of 12 experiments. Open bars: control macrophages; filled bars: secretions-differentiated macrophages. Values are significantly (*$p<0.05$) different from those for control macrophages.

Discussion

The main conclusion from the present study is that maggot secretions skew the monocyte-macrophage differentiation away from a pro-inflammatory to a pro-angiogenic type. This conclusion is based on the following observations. First, maggot secretions dose-dependently led to MØ-1 producing less IL-12p40, TNF-α and MIF upon LPS stimulation as compared to control cells. Similar results were obtained for MØ-2 upon stimulation with low amounts of LPS. These actions of maggot secretions on the differentiation of macrophages are not limited to stimulation via TLR-4 as similar effects were observed when stimulated via the TLR-2 pathway. Interestingly, adding secretions (35 µg/mL) to fully differentiated macrophages did not lead to a reduced production of IL-12p40 or TNF-α (data not shown), indicating that secretions effect the differentiation of the cells. Second, maggot secretions led to MØ-1 and MØ-2 with a reduced production of the chemokines MIP-1β, RANTES and PDGF-BB and an increased production of MCP-1 and IL-8. Based upon these findings, it is not possible to predict the overall effect of maggot secretions on migration of leucocytes into the inflamed site. However, earlier we reported that secretions reduce the migration of both monocytes[15] and neutrophils[14] irrespective of the presence of chemokines. Therefore, migration of leucocytes will likely be reduced in the presence of secretions. Third, secretions dose-dependently led to MØ-2, but not MØ-1, with enhanced production of bFGF and VEGF. These growth factors, together with IL-8, are involved in endothelial cell migration and proliferation which is essential for angiogenesis[20,26]. In addition, low amounts of TNF-α, as observed after exposure of secretions-differentiated macrophages to LPS, are known to induce angiogenesis as well. The exact roles of the elevated IL-6 production by secretions-differentiated macrophages in inflammation and neovascularisation are unclear as this cytokine often exerts its effects by regulating the production of other molecules, such as MIP-1[27], which we did not observe.

Other findings pertain to the effect of maggot secretions on monocyte-macrophage differentiation with regard to the expression of cell-surface receptors. First, secretions led to MØ-1 and MØ-2 with increased expression of TLR2 and TLR4, as compared to control cells. Additionally, the expression of the mannose receptor CD206 was increased by secretions-differentiated MØ-1 while the CD14 expression was still detectable on these cells. These results suggest that secretions-differentiated macrophages may become more sensitive to pathogen-associated molecular patterns, like LPS and LTA. However, we found no enhanced sensitivity of the cells to these stimuli. Consequently, our results may be caused by interference of secretions with signal transduction pathways down stream of receptor activation such as a transient rise in cAMP[28,29] and is reported for monocytes[15] and neutrophils[14] after exposure to secretions. Second, secretions differentiated MØ-1 and MØ-2 displayed enhanced expression of CD16. Additionally, the expression of CD11b (part of CR3), involved in both phagocytosis and adhesion to endothelial cells[30], was enhanced on

secretions differentiated MØ-1. Expression of CD35 (CR1) was enhanced only on naïve MØ-1. Together, it will be of interest to investigate whether the increased expression of above mentioned receptors mediate phagocytosis of pathogens by macrophages. Third, secretions led to MØ-1 with decreased expression of the co-stimulatory molecule B7.2. This may indicate a reduction in MØ-1-induced Th1 cell proliferation and function[31,32]. As MØ-2 do not support Th1 cell activation, the effect of the secretions-induced decreased expression of B7.2 and HLA-DR on these cells is not clear. Together, maggot secretions may effect macrophage T cell interactions and this will be the subject of further studies.

Another remarkable finding of this study pertains to the differential effects of LPS on MØ-1 and MØ-2. LPS stimulates a pro-inflammatory responses in MØ-1 and subsequently down-regulates the expression of TLR2 and TLR4 on these macrophages, which is reported earlier as LPS tolerance[33]. These tolerized MØ-1 poorly respond to another challenge with LPS, thus reducing the pro-inflammatory response and preventing excessive reactions against infection and subsequent detrimental effects on the surrounding tissue. In contrast, MØ-2 exert anti-inflammatory and pro-angiogenic activities. However, once the infection is cleared these cells may have to initiate a swift response against a starting/recurring infection. Therefore, the increased expression of TLRs on these cells may act as a positive regulator of inflammation. In agreement, we found that MØ-2 produce relatively high levels of pro-inflammatory cytokines upon stimulation with low, physiological amounts of LPS (0.01 or 0.1 ng of LPS/mL) as compared to high levels of LPS (100 ng/mL). In addition, MØ-2 produce high levels of chemokines upon LPS stimulation indicating that these cells can attract many additional immune cells. Collectively, it would be interesting to further investigate the differences between these two subsets of macrophages and their role in acute and chronic wounds.

What could be the relevance of the present findings? In a normal wound healing process, resident cells like macrophages efficiently detect microbial structures and respond to this by recruiting neutrophils and monocytes to fight off the invading pathogens. Initially, monocytes may differentiate to pro-inflammatory macrophages that regulate the inflammatory process. When the infection recedes due to removal of pathogens and cellular debris, the composition of the local environment will change facilitating differentiation of monocytes to anti-inflammatory/pro-angiogenic macrophages. These cells suppress inflammatory responses directly[19,34,35] and indirectly by inducing regulatory T cells[36] and mediate neovascularisation, cell proliferation[20] and subsequent matrix synthesis[21,22] resulting in repair of the wound. In agreement, we found MØ-1 to produce considerable levels of pro-inflammatory cytokines (e.g. TNF-α, IL-12p40, MIF) whereas MØ-2 produced high levels of IL-10, bFGF and VEGF. Although pro-inflammatory cytokines are essential for acute inflammatory responses, they can be detrimental in chronic wounds were inflammation persists. Histological data exists showing that parts of chronic wounds seem to be stuck in different phases of healing, with loss of synchronicity, which is essential for a

rapid healing[37]. Some parts of the wound that are ready for fibroblast proliferation and epidermal resurfacing could be damaged by the inflammatory phase still present in other parts of the wound[38]. Chronic leg ulcers are associated with elevated expression of pro-inflammatory cytokines, like TNF-α and MIF, compared to acute wounds[39-41]. These cytokines promote the production of more pro-inflammatory cytokines[42,43], up-regulate the synthesis of matrix metalloproteinases and serine proteases[24,42,44] and activate the reactive oxygen generating system[45,46]. Together, these pro-inflammatory actions result in extracellular matrix destruction[47-49] and inactivation of growth factors and protease inhibitors[44,50-52]. Our results showed inhibited production of pro-inflammatory cytokines by macrophages differentiated in the presence of secretions. These actions of maggots may provide protection against progression towards ongoing inflammation and tissue destruction by these cells in chronic wounds and may result in an environment beneficial for healing. Simultaneously, the increased pro-angiogenic activity of anti-inflammatory macrophages may induce neovascularisation and the concurrent formation of granulation tissue. In agreement, others reported that maggots increase the expression of bFGF in ulcer tissue[53] and induce the formation of granulation tissue[9,54]. Taken together, the actions of secretions described in this study contribute to the exiting beneficial effects of maggots in diabetic foot ulcers and other chronic wounds unresponsive to conventional therapies.

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CHAPTER EIGHT

General discussion and Summary

Non-healing, chronic wounds often result in a reduced quality of life for patients, due to decreased physical, emotional and social function and are a major economic cost for both the patients, their families and Society as a whole[1]. These wounds are particularly prone to occur in patients suffering from acute, extended trauma as well as in patients with vascular insufficiencies and underlying chronic conditions such as diabetes mellitus. Although there are numerous reasons for the development of a non-healing chronic wound, one of the major mechanisms underlying failure of healing is a prolonged and unregulated inflammatory response (**Chapter 1**). Whereas many therapies have been developed to address the problematic healing of these wounds, maggot therapy may be the most successful one, having a success rate around 7 out of every 10 wounds unresponsive to conventional therapies. Some characteristics (e.g. obesity, smoking, diabetes mellitus, wound duration and size) were not contra-indicative with respect to eligibility for maggot therapy, whereas others (chronic limb ischaemia, wound depth, and age) negatively influenced the outcome[2]. Clearly, the modes of action of maggot therapy likely involve multiple wound healing processes. The studies reported in this thesis focussed on the effects of maggot excretions/secretions on processes related to the inflammatory phase of wound healing. The findings are summarised in Figure 1.

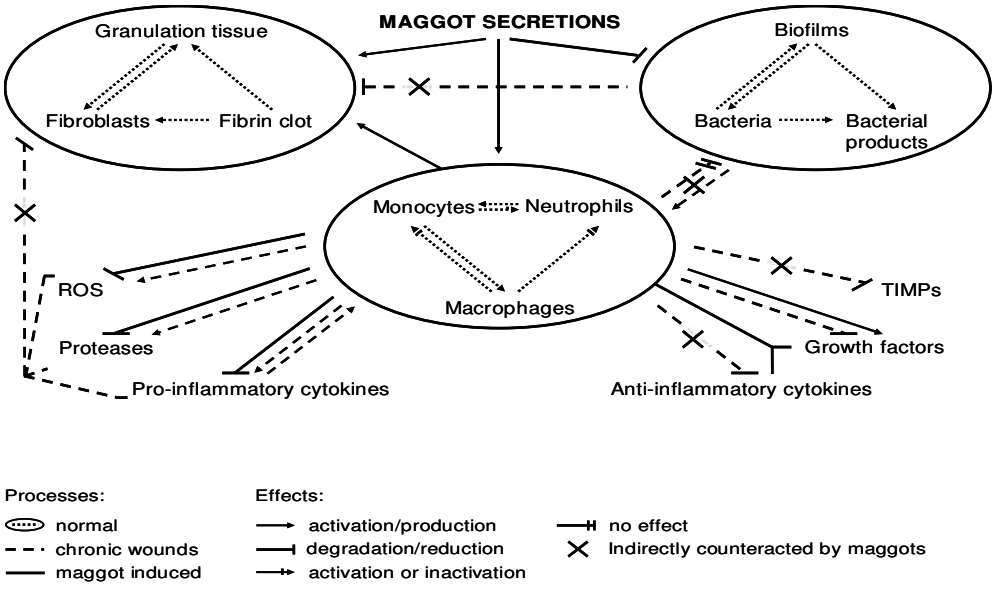

Figure 1 Effects of maggot secretions on cells, cellular products and processes associated with chronic wounds. ROS, reactive oxygen species; TIMPs, tissue inhibitor of metalloproteinases.

Maggot excretions/secretions combat bacterial infections

A major complication of wound healing is the occurrence of bacterial infections[3-5], especially when the bacteria reside in biofilms[6] which protect them from the actions of cells and molecules of the immune system[5,7] and antibiotics[8,9]. Moreover, we found some antibiotics (vancomycin, daptomycin, and flucloxacillin) enhance *S. aureus* biofilms, whereas other antimicrobial drugs (clindamycin and linezolid) were unable to totally eradicate the biofilms (**Chapter 3**). Consequently, bacterial re-growth may arise from the remaining biofilms and could be an explanation for the persistence of infections often encountered in chronic wounds. One of the beneficial effects of maggot excretions/secretions (ES) is the ability to inhibit the formation and breakdown of biofilms of *S. aureus* (**Chapter 2**). This biofilm breakdown occurred irrespective the presence of antibiotics (**Chapter 3**). In addition, ES broke down biofilms of *P. aeruginosa,* when using 10-fold higher doses of ES than the effective concentrations used against *S. aureus* biofilms, whereas low concentrations of ES enhanced biofilm formation by these pathogens (**Chapter 2**). Others have shown that, *in vitro, P. aeruginosa* but not *S. aureus,* impairs maggot survival[10]. Based on these findings and as suggested by clinical experience[11], we conclude that more maggots should be applied to make treatment successful for wounds colonized and infected with *P. aeruginosa,* as compared to those by *S. aureus*. As secretions interfered in a similar fashion with the TLR-2 and TLR-4 triggered intracellular pathways of monocytes (**Chapter 5**) and pro-inflammatory macrophages (**Chapter 6**), it is unlikely that a differential modulation of cell responses by Gram-positive and Gram-negative bacteria is the cause of the observed differences in effects of maggots between patients with wounds infected with *S. aureus* and *P. aeruginosa*.

Interestingly, disruption of bacterial biofilms by ES does not involve the killing of bacteria as the micro-organisms released from the biofilm remained viable (**Chapter 2**). However, several reports describe bactericidal properties of ES against planktonic bacteria[12-14]. The reason for this apparent discrepancy is that the concentration of ES effective against biofilms is considerably lower than those needed to kill the bacteria. Moreover, this level of ES is not within the range achieved in wounds during maggot therapy. Therefore, maggots cannot be considered as a replacement for antibiotics, but should be used only as a supplementary treatment. Of note, it has been reported that antibiotics do not affect the viability of maggots[15]. The consequence of biofilm breakdown is that bacteria will become subject to the actions of antibiotics and the immune system as well as to ingestion and subsequent degradation by maggots[16,17].

Unexpectedly, we initially observed that antibiotics were inactive or less effective against the bacteria that were released from *S. aureus* biofilms. We argued that such bacteria are initially resistant to antibiotics due to their metabolic state; once the bacteria started to multiply, they became more susceptible to antimicrobial action (**Chapter 3**). In agreement with this, daptomycin, which acts on dormant (static) and exponentially growing bacteria,

was the most active antibiotic in this respect. Moreover, ES increased the activity of daptomycin against bacteria derived from biofilms. Taken together, it can be stated that maggot secretions breakdown bacterial biofilms, thereby subjecting the released bacteria to the action of antibiotics and the immune system. As a result, the unopposed stimulation of the inflammatory response by the bacterial products released from bacteria within biofilms may come to a halt.

Maggot secretions regulate inflammatory responses

Maggot secretions did not affect the ability of neutrophils and monocytes to phagocytose and intracellularly kill bacteria (**Chapter 5 and 6**). The two main mechanisms involved in bacterial killing by neutrophils are the production of reactive oxygen species (ROS) and degranulation, i.e. the release of enzymes, antimicrobial peptides etc into the phagolysosome containing the micro-organisms. H_2O_2 is the most stable ROS and as elastase is a very destructive enzyme[18,19], we therefore focussed on the effects of maggot secretions on the production and release of these molecules by neutrophils. Our results show that secretions dose-dependently inhibit these activities in response to the chemotactic peptide fMLP and the protein kinase C activator PMA (**Chapter 5**).

Besides clearing infections, monocytes and especially macrophages, play a major role in regulating cellular behaviour and other processes in the wounds[20]. We therefore investigated the effect of secretions on the production of pro-inflammatory cytokines by these cells. Maggot secretions reduced the LPS-induced production of several pro-inflammatory cytokines by monocytes as well as that by cells in whole blood (**Chapter 6**). Similar findings apply to pro-inflammatory macrophages that differentiated from monocytes (i.e. as induced by growth factors) in the presence of the secretions (**Chapter 7**). Taken together, maggot secretions reduce the production and/or release of pro-inflammatory mediators by phagocytes, thereby contributing to the inhibition of pro-inflammatory activity in chronic wounds. The observed effects of secretions on cell functions are unlikely to be based in altered expression profiles as maggot secretions induced different and/or contrasting effects on the expression of cell surface receptors on neutrophils (**Chapter 5**), monocytes (**Chapter 6**) and macrophages (**Chapter 7**).

Pro-inflammatory macrophages are also responsible for the recruitment and activation of Th1 lymphocytes, through cytokine production, *via* expression of co-stimulatory molecules and by antigen processing and presentation[21,22]. These T-cells in turn induce activation of pro-inflammatory macrophages, thereby enhancing their pro-inflammatory responses. Since maggot secretions inhibit the production of pro-inflammatory cytokines and reduce the expression of the T-cell co-receptor CD86 on macrophages (**Chapter 7**), Th1 cell proliferation and function may be reduced. Furthermore, preliminary experiments showed secretions to decrease the IFN-γ production by T-cells in whole blood stimulated with monoclonal antibodies directed against CD3 and CD28 for 24 h (unpublished observations).

By contrast, using a different experimental set-up, we observed an increased production of IFN-γ when stimulating the cells with mAbs against CD2 and CD28. Clearly, the effects of secretions on T-cells should be investigated further as no conclusions can be obtained from the present data.

Maggot secretions inhibit migration of phagocytes

Apart from components and products released by bacteria, chemokines released at the site of inflammation can attract large numbers of inflammatory cells[23-25]. Therefore, we investigated whether maggot secretions influenced migration and chemokine production by phagocytes. The results revealed that secretions altered the production of several chemokines by monocytes (**Chapter 6**). Similar effects were observed using macrophages differentiated from monocytes in the presence of secretions (**Chapter 7**). Using supernatants of monocyte-cultures incubated with secretions, we observed reduced monocyte chemotaxis, as compared to supernatants of control cultures. However, secretions dose-dependently inhibited the migration of both neutrophils (**Chapter 5**) and monocytes (**Chapter 6**) towards fMLP directly, making the changes in chemokines of an overall lesser importance regarding the outcome. Thus, inhibition of leucocyte migration by maggot secretions may contribute to reduced pro-inflammatory responses in chronic wounds.

Wound matrix and debridement

Although maggot excretions/secretions breakdown bacterial biofilms and suppress pro-inflammatory responses of phagocytes, these effects may be insufficient to reverse an impaired wound healing. When the wound has been infected for a considerable time, the actions of the pathogenic bacteria and/or the immune cells combined likely have led to the destruction of the provisional matrix (and also the surrounding healthy tissue), which then no longer supports re-epithelialisation and granulation tissue formation (**Chapter 1**). This means that the corrupted tissue has to be removed. This cannot be accomplished by the wound components alone as the lysis of fibrin clots (fibrinolysis) may be impaired in chronic wounds, due to enhanced levels of the fibrinolysis inhibitor PAI. We found enhanced plasminogen activator-induced fibrinolysis by a serine protease present in the secretions of maggots (**Chapter 4**). Consequently, suboptimal levels of plasminogen activators may be sufficient for effective fibrinolysis in chronic wounds. Interestingly, secretions were unable to dissolve plasma clots. These findings are in contrast to those of another report that described the use of a ‘clot’ composed of fibrin only[26]. We therefore concluded that the clot composition may be an important factor for the activity of the enzymes within maggot secretions. Together, wound debridement by maggots may involve a combined action of fibrinolytic activity of the wound components and enzyme activity within the secretions.

Granulation tissue formation

Clinical observations indicate that maggots induce the formation of granulation tissue[27,28]. This could result from debridement in combination with suppression of pro-inflammation and clearance of the bacteria. However, we found that maggot secretions enhanced the production of the growth factors VEGF and bFGF by monocytes (unpublished observations) and pro-angiogenic macrophages (**Chapter 7**). In agreement with this earlier reports observed secretions-induced enhanced levels of bFGF in ulcer tissues[29]. Growth factors, IL-8 and low levels of TNF-α are involved in endothelial cell migration and proliferation which are essential for angiogenesis[30,31]. Moreover, our preliminary data showed elevated IL-8 levels in fluid samples from wounds treated with maggots for 3 or 4 days, as compared to fluids obtained from these wounds just before the start of the therapy (unpublished observations). Thus, the increased pro-angiogenic activity may induce neovascularisation and the concurrent formation of granulation tissue.

Active components within maggot excretions/secretions

Maggot excretions/secretions contain a wide variety of components that may induce various effects on human cells and the processes involved in wound healing. The results in Chapters 4, 6 and 7 were obtained with maggot secretions whereas the less 'pure' mixtures of excretions/secretions were used in the experiments described in Chapters 2, 3 and 5. Additional experiments showed that maggot secretions breakdown biofilms as well (unpublished observations). Although we did not test the effect of secretions on neutrophils, we assume that the active component is identical to the one responsible for the actions of maggot secretions on monocytes and macrophages.

During our attempts to isolate and characterise the active components within maggot excretions/secretions we gained some knowledge on this topic. First of all, the breakdown of *S. aureus* biofilms was facilitated by heat-sensitive molecules (enzymes) within ES (**Chapter 2**). By contrast, heat-resistant molecules affected *P. aeruginosa* biofilms. Maggots produce a wide variety of molecules with antimicrobial activity. Using gel-filtration and RP-HPLC, we isolated a small number of peptides/proteins from the haemolymph of maggots that potentially exert antimicrobial activity (unpublished observations). These molecules were also present in the excretions/secretions of the maggots. Chromatographic techniques and mass spectrometry, together with functional assays, revealed that the active component of maggot secretions enhancing fibrinolysis was a trypsine-like serine protease. The component in the secretions that affects phagocytes remains to be elucidated. As also observed for *P. aeruginosa* biofilms, the active molecule did not bind to a C18-RP-HPLC column indicating that it is not a peptide/protein (unpublished observations). By contrast, we were able to reveal aspects of the mechanism by which secretions may affect phagocytes. Within 15 sec after the addition to the cells, maggot secretions maximally increased the intracellular concentration of cAMP both in neutrophils (**Chapter 5**) and monocytes (**Chapter**

6). The cAMP signalling cascade can lead to a range of immunomodulatory effects on cells such as neutrophils, monocytes, macrophages and T lymphocytes[32]. It has been reported that activation of cAMP pathways is associated with a reduced production of pro-inflammatory cytokines while enhancing the production of IL-10 and VEGF[32-34]. Furthermore, elsewhere it has been reported that a rise in cAMP is involved in a decreased migration[35,36], degranulation and respiratory burst[37,38] while inhibiting apoptosis in several cell types[39-41]. Overall it can be concluded that the effects of maggot secretions on phagocytes is explainable on the basis of elevated intracellular cAMP levels.

Therapeutic considerations

Maggots are applied to wounds using either a 'free-range' or a 'contained' (biobag) technique. It has been reported that debridement by free-ranging maggots but not maggots in biobags, can lead to bleeding of wounds[42]. The most widely accepted explanation is that crawling of the maggots can cause bleeding[43] although there is a lack of scientific evidence to support this view. It is also possible that enzyme activity of maggots and enhanced fibrinolysis result in breakdown of the provisional matrix before the underlying tissue is healed. In this context, it is likely that a large part of the secretions stick to the biobags, thereby lowering the level of active molecules in the wounds compared to that obtained with free-ranging maggots. In agreement with this, it is reported that free-ranging maggots are more successful than maggots in biobags[44].

We found that maggot secretions affect a broad range of processes related to chronic wounds. Based on these results, it may be possible to replace maggots by their secretions thereby eliminating the so-called 'yuk-factor' which plays a negative role in the acceptance of maggot therapy.

Summary and Conclusion

Maggot excretions/secretions breakdown biofilms of both Gram-positive and Gram-negative bacteria, exposing them to the immune system, antibiotics, and ingestion and subsequent degradation by the maggots. Furthermore, proteases in maggot secretions enhance debridement by increasing the fibrinolytic activity of wound components and by degrading matrix components directly. Additionally, maggot secretions inhibit the pro-inflammatory responses of phagocytes but do not affect their ability to ingest and intracellularly kill micro-organisms. Finally, secretions induce the production of growth factors essential for angiogenesis. Clearly, all these effects may be beneficial for the recovery of chronic wounds. The reason why maggots exert these effects may well be a simple matter of survival. Similar to other multi-cellular organisms, maggots have evolved ways to deal with detrimental bacteria, using antimicrobial molecules and, in case of biofilms, with enzymes and other additional mechanisms. Furthermore, maggots obtain their nutrients from dead tissue. To digest the tissue remnants they produce numerous enzymes. In addition, common

mechanisms for parasitic organisms to successfully invade the host are by inducing fibrinolysis and/or suppressing the host immune system, since inflammatory responses of the cells from the host may be detrimental to survival of the parasite. Clearly, maggots do not harm their human hosts. However, uncontrolled myasis, as observed in some cattle, can become lethal and should therefore be avoided.

In conclusion, the results described in this thesis provide new insights into the modes of action of maggot therapy in chronic wounds. The success of maggot therapy may be explained by the broad spectrum of processes that are modulated by maggot secretions. These results contribute to further acceptance of this efficient and successful therapy for the treatment of chronic wounds.

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CHAPTER NINE

Nederlandse samenvatting

Chronische (niet-helende) wonden zijn wereldwijd een groot maatschappelijk probleem. De patiënten kunnen door hun fysieke achteruitgang emotionele problemen krijgen en sociaal geïsoleerd raken. Dit leidt tot een verminderde kwaliteit van leven en kan gepaard gaan met hoge kosten voor de patiënten, maar ook voor hun families en de samenleving. Chronische wonden kunnen worden veroorzaakt door zowel lokale als algemeen lichamelijke factoren. Voorbeelden van lokale factoren zijn bacteriële infecties, zuurstofgebrek, verweking van het weefsel, eeltvorming en druk (bijvoorbeeld doorligwonden) terwijl algemeen lichamelijke factoren te maken hebben met slechte voeding, leeftijd, vaatlijden, immuunsysteemonderdrukkende medicijnen en onderliggende ziektes. Een voorbeeld van een onderliggende ziekte is diabetes mellitus. Van de 200 miljoen diabeten in de wereld krijgt tot wel 15% te maken met één of meerdere niet-helende voetzweren, die leiden tot meer dan 1 miljoen amputaties per jaar. Chronische wonden komen vaak voor in de onderbenen van mensen ouder dan 60. De verwachting is dat het aantal patiënten zal toenemen.

Er zijn veel verschillende manieren om chronische wonden te behandelen. Verwijdering van beschadigd, geïnfecteerd en/of dood weefsel (debridement) is één van deze behandelingsmethoden en kan worden uitgevoerd door maden van de groene vlieg. Het gebruik van maden voor de genezing van wonden is waarschijnlijk al eeuwenoud en wordt bijvoorbeeld toegeschreven aan de maya's en de aboriginals. De oudst bekende tekst die melding maakt van de positieve effecten van maden in wonden is geschreven door Baron D.J. Larrey (1766-1842), inspecteur-generaal in het leger van Napoleon. Verdere bronnen komen van chirurgen die de gunstige effecten van maden op wonden beschreven ten tijde van de Amerikaanse Burgeroorlog. De grondlegger van de moderne madentherapie is de orthopedisch chirurg William Baer (1872-1931). Tijdens de Eerste Wereldoorlog behandelde hij soldaten, die van het slagveld waren gehaald, waarvan de wonden vol zaten met maden. Toen hij de maden verwijderde bleek dat de wonden heel schoon waren. In 1928 startte Baer met de behandeling van wonden met maden en hij ontwikkelde manieren om maden te kweken en te steriliseren. In de 30-er jaren werd madentherapie een zeer populaire behandelingsmethode, wat af te leiden is aan de vele wetenschappelijke artikelen over dit onderwerp. Door de ontwikkeling van verbeterde chirurgische methoden en de ontdekking van antibiotica stopte het gebruik van maden en raakte deze therapie vergeten. In de jaren 90 werd madentherapie echter opnieuw geïntroduceerd als behandeling van chronische wonden besmet met antibiotica-resistente bacteriën. Tegenwoordig wordt deze therapie over de hele wereld weer gebruikt. In Nederland is madentherapie eens of meerdere keren toegepast in 70% van alle ziekenhuizen terwijl 25% zelfs regulier gebruiker is. Er zijn echter geen goede richtlijnen voor het gebruik van maden en de gepubliceerde literatuur is vaak onsamenhangend en/of onwetenschappelijk. Verder is er zeer weinig bekend over het werkingsmechanisme van de therapie. Hierdoor is madentherapie bij veel artsen nog niet echt geaccepteerd.

Het doel van het beschreven onderzoek in dit proefschrift is de werkingsmechanismen van madentherapie te verklaren. Hiertoe bekijken we het effect van madensecreten (speeksel) op 1) bacteriën, 2) de vorming en afbraak van bloedstolsels, 3) op de werking van verschillende soorten witte bloedcellen (neutrofielen en monocyten) en 4) op de ontwikkeling van monocyten tot ontstekingsbevorderende macrofagen en ontstekingsremmende/ herstelbevorderende macrofagen.

Hoofdstuk 1 geeft een samenvatting van normale wondgenezingsprocessen. Deze processen kunnen worden verdeeld in drie overlappende fasen: ontsteking, weefselvorming, en herstructurering van nieuw weefsel. Verder worden de stoornissen in deze fasen beschreven die het ontstaan van chronische wonden kunnen verklaren en worden verschillende behandelingsmethoden voor deze wonden genoemd. Ten slotte wordt een overzicht gegeven van de geschiedenis van madentherapie.

Eén van de effecten die wordt toegeschreven aan maden is het doden van bacteriën door middel van de productie van antimicrobiële moleculen en enzymen. Om dit te bewijzen hebben we de antimicrobiële activiteit van madensecreten met behulp van verschillende methoden onderzocht (**Hoofdstuk 2**). We vonden echter dat de bacteriën niet gevoelig waren voor de secreten; slechts bij concentraties die aanzienlijk hoger waren dan in wonden verwacht wordt, zagen we een afname van het aantal *Staphylococcus aureus*, maar niet van *Pseudomonas aeruginosa* (twee veelvoorkomende bacteriën in chronische wonden). Bacteriën komen echter niet alleen als vrije cellen voor in chronische wonden, maar ook in biofilms. Biofilms zijn een samenleving van bacteriën die vastzitten aan een substraat of aan elkaar en zijn omgeven door een zelfgeproduceerde matrix. De bacteriën in deze biofilms zijn beschermd tegen zowel antibiotica als de werking van het immuunsysteem. Dit komt doordat de bacteriën hun metabolisme verlagen (inactief worden) en door de aanwezigheid van de beschermende matrix. We ontdekten dat madensecreten de vorming van *S. aureus* biofilms kunnen voorkomen en dat ze al gevormde biofilms kunnen afbreken (**Hoofdstuk 2**). In tegenstelling tot *S. aureus* biofilms kan de vorming van *P. aeruginosa* biofilms niet worden voorkomen door secreten. Wel vonden we dat relatief hoge concentraties secreten de gevormde biofilms konden afbreken, terwijl lage concentraties leidden tot vergroting van de biofilms. Verder is bekend dat *S. aureus* geen effect heeft op het overleven van de maden, terwijl *P. aeruginosa* maden kan doden. Daarom is onze conclusie dat meer maden nodig zijn voor de behandeling van wonden die geïnfecteerd zijn met *P. aeruginosa* dan met *S. aureus*. De bacteriën uit de biofilms werden echter niet gedood door de madensecreten. Daarom onderzochten we het effect van combinaties van antibiotica en secreten op biofilms en op de bacteriën die uit de biofilms kwamen (**Hoofdstuk 3**). We ontdekten dat de afbraak van *S. aureus* biofilms door secreten niet veranderde in de aanwezigheid van antibiotica. De bacteriën die uit de biofilm kwamen werden echter niet efficiënt gedood door de antibiotica. Dit komt waarschijnlijk doordat de bacteriën niet actief waren; zodra de bacteriën in vers

medium werden overgebracht en begonnen te groeien konden ze worden gedood door de antibiotica. Wel zagen we een groot verschil in de effectiviteit van de antibiotica. Clindamycin en vancomycine, antibiotica die alleen effectief zijn tegen groeiende cellen, zorgden voor maximaal 99% afname van het aantal bacteriën, terwijl daptomycine, een antibioticum dat zowel tegen groeiende als inactieve cellen werkt, veel sneller werkte en uiteindelijk alle bacteriën doodde. Verder ontdekten we dat madensecreten de werking van daptomycine tegen bacteriën uit biofilms verhoogde. Samenvattend, madensecreten breken biofilms af. De vrijgekomen bacteriën zijn echter niet actief en kunnen daarom alleen gedood worden door specifieke typen antibiotica, zoals daptomycine. Omdat secreten de bacteriën zelf niet kunnen doden, mogen maden nooit gebruikt worden ter vervanging van antibiotica (wat soms wel wordt gedaan). In plaats hiervan zouden maden en antibiotica samen moeten worden gebruikt om infecties te bestrijden. Dit is ook belangrijk, omdat we ontdekten dat sommige typen antibiotica (vancomycine, daptomycine en flucloxacilline) ervoor zorgden dat biofilms van *S. aureus* juist groter werden, terwijl andere antibiotica (clindamycine en linezolid) de biofilms slechts gedeeltelijk afbraken (**Hoofdstuk 3**). In alle gevallen blijven de bacteriën aanwezig en kunnen weer uitgroeien en opnieuw een infectie veroorzaken.

Naast antibiotica kunnen bacteriën ook gedood worden in het spijsverteringskanaal van de maden en door bepaalde typen witte bloedcellen. We ontdekten dat neutrofielen (**Hoofdstuk 5**) en monocyten (**Hoofdstuk 6**) net zo effectief waren in het opruimen van bacteriën in de aanwezigheid van madensecreten als in de afwezigheid.

Bacteriën en bacteriële producten leiden tot de migratie en activatie van witte bloedcellen. In normale wonden is dit belangrijk om de infectie te bestrijden. In chronische wonden kunnen deze ontstekingsreacties echter weefselbeschadiging veroorzaken, mede doordat witte bloedcellen de biofilms niet goed kunnen bestrijden en daardoor de ontstekingsreactie ontspoord raakt. Daarom hebben we onderzocht wat het effect van maden is op deze processen. We ontdekten dat madensecreten de migratie van neutrofielen (**Hoofdstuk 5**) en monocyten (**Hoofdstuk 6**) remden. Secreten zorgden ook voor een verandering in de productie van migratiebevorderende moleculen (chemokines) door monocyten (**Hoofdstuk 6**) en macrofagen (**Hoofdstuk 7**). Dit veranderde de migratieremming echter niet.

Witte bloedcellen kunnen bacteriën doden met behulp van zuurstofafhankelijke en -onafhankelijke mechanismen. Ook al zagen we geen verandering in het aantal en de snelheid waarmee bacteriën gedood werden door de cellen, de twee mechanismen werden wel onderdrukt door de maden in neutrofielen (**Hoofdstuk 5**). Monocyten en de daaruit voortkomende macrofagen zijn ook belangrijk voor de regulatie van wondprocessen door middel van de productie van ontstekingsbevorderende en -remmende moleculen. Omdat in chronische wonden de balans tussen deze moleculen is verstoord, hebben we onderzocht wat de effecten zijn van madensecreten op de productie van bepaalde ontstekings-

bevorderende moleculen (cytokines) door de cellen. We ontdekten dat de productie van deze cytokines door gezuiverde monocyten, door cellen in volbloed en door ontstekingsbevorderende macrofagen werd geremd in de aanwezigheid van secreten (**Hoofdstuk 6 en 7**). Het uiteindelijke resultaat van het onderdrukken van celmigratie en ontstekingsreacties in chronische wonden is vermindering van weefselbeschadiging door de witte bloedcellen.

De aanwezigheid van bacteriën in de wond en de daarmee samenhangende ontstekingsreacties leiden tot beschadiging van de aanwezige bloedstolsels en het omringende gezonde weefsel. Als gevolg hiervan kunnen er geen nieuwe huid en onderliggende weefsels worden gevormd. Daarom moet het aangetaste weefsel worden verwijderd. Dit kan echter niet door het lichaam zelf worden gedaan omdat in chronische wonden het proces dat verantwoordelijk is voor de verwijdering van bloedstolsels, fibrinolyse genaamd, vaak niet goed werkt door de aanwezigheid van een overmaat aan fibrinolyseremmers ten opzichte van de activatoren. We ontdekten dat madensecreten een specifiek enzym bevatten, een serine protease, die de werkzaamheid van het fibrinolyse proces verhoogt (**Hoofdstuk 4**). De reden hiervoor is dat dit enzym een interactie aangaat met plasminogeen (een essentiële fibrinolysecomponent) waardoor de afgenomen hoeveelheden van de activatoren alsnog voldoende zouden kunnen zijn om de fibrinolyse te activeren. Madensecreten hadden geen effect op de vorming van bloedstolsels en de enzymen in madensecreten konden de gevormde bloedstolsels niet afbreken. Het is waarschijnlijk wel zo dat de secreten de aangetaste weefsels in chronische wonden kunnen afbreken.

Het bestrijden van de bacteriën, het remmen van de ontstekingsreactie en het verwijderen van aangetast weefsel door maden leiden gezamenlijk tot bevordering van het wondherstelproces. We ontdekten echter dat de secreten dit proces ook direct beïnvloeden door middel van de verhoging van de productie van groeifactoren door monocyten en ontstekingsremmende/herstelbevorderende macrofagen (**Hoofdstuk 7**). Deze groeifactoren zijn belangrijk voor de vorming van nieuwe bloedvaten en weefsels.

De resultaten in dit proefschrift laten zien dat maden verschillende effecten hebben die verstoorde processen in chronische wonden kunnen onderbreken en de balansen kunnen herstellen. Het is daarom interessant om te onderzoeken welke moleculen in de secreten van de maden verantwoordelijk zijn voor deze effecten. Zoals alle dieren produceren maden een grote variëteit aan antimicrobiële moleculen. Met behulp van verschillende technieken hebben we een aantal peptiden/eiwitten geïsoleerd uit de maden die potentieel antimicrobieel zijn. Een deel van deze moleculen, die nog nagemaakt en verder getest moeten worden, is ook aanwezig in de secreten. Verder vonden we dat de afbraak van *S. aureus* biofilms werd veroorzaakt door hittegevoelige moleculen, waarschijnlijk enzymen, terwijl hitteongevoelige moleculen leidden tot de afbraak van *P. aeruginosa* biofilms. De

actieve moleculen in de secreten die verantwoordelijk zijn voor het ontstekingremmende effect op witte bloedcellen zijn tot nu toe onbekend. We weten echter wel hoe de actieve componenten deze cellen beïnvloeden, namelijk door verhoging van cyclisch AMP (**Hoofstuk 5 en 6**). Dit 'boodschappermolecuul' is verantwoordelijk voor de remming van ontstekingsprocessen in de cellen.

Madensecreten breken biofilms af waardoor de bacteriën gevoelig worden voor antibiotica, het immuunsysteem en voor digestie door maden zelf. Bovendien produceren de maden enzymen die het fibrinolyseproces bevorderden en ervoor zorgen dat dood weefsel wordt opgeruimd. Verder onderdrukken maden de ontstekingsreacties van witte bloedcellen zonder dat het opruimen van bacteriën door deze cellen wordt verstoord. Ten slotte wordt de productie van groeifactoren juist bevorderd door de secreten. Al deze effecten zijn positief voor de heling van chronische wonden. De vraag die dan overblijft is waarom maden dit doen. Het antwoord op deze vraag is eigenlijk heel simpel…overleven. Zoals voor alle organismen geldt, kunnen bacteriën schadelijk of zelfs dodelijk zijn voor maden. Daarom hebben maden mechanismen ontwikkeld om de bacteriën te bestrijden. Verder leven maden van dood weefsel. Door middel van de productie van veel verschillende enzymen zijn maden in staat om dit weefsel te verteren. Verder is het niet ongewoon voor parasieten om het immuunsysteem van de gastheer te onderdrukken, aangezien ontstekingsreacties schadelijke effecten kunnen hebben. Ook al zijn maden dus eigenlijk parasieten, ze beschadigen wonden in de mens niet. Dit heeft te maken met het type maden. Medische maden produceren factoren die voornamelijk dood weefsel aantasten (saprofage maden). Het is echter wel bekend bij dieren, bijvoorbeeld schapen, dat ongecontroleerde aanwezigheid van dit type maden in wonden tot ziekte en uiteindelijk tot de dood kan leiden. Daarom moet de therapie altijd worden toegepast door goed opgeleide artsen. Uit onze resultaten blijkt dat de secreten van de maden de componenten bevatten die werkzaam zijn in de wond. Het is daarom mogelijk dat de maden uiteindelijk kunnen worden vervangen door de secreten, of zelfs beter, door actieve moleculen die geïsoleerd zijn uit de secreten. Tot slot, er is geen enkele therapie die alle chronische wonden kan genezen; ook maden niet. Maden beïnvloeden echter veel verschillende processen in wonden en dit is waarschijnlijk de reden van het succes van madentherapie.

Curriculum vitae

Mariena van der Plas werd geboren op 24 december 1980 te Leiden. Na het behalen van het VWO diploma in 1999 aan het Andreas College locatie Pieter Groen te Katwijk begon ze met de studie Biologie aan de Universiteit van Leiden. In 2000 haalde Mariena haar propedeuse *cum laude* en koos ze voor de specialisatierichting Moleculaire Biologie en een jaar later ook voor de richting Medische Biologie. In het kader van deze specialisaties liep ze stage bij de afdeling Infectieziekten onderleiding van Peter H. Nibbering. In samenwerking met Martine de Jager van de afdeling oogheelkunde startte ze het onderzoek naar een mogelijke associatie tussen het krijgen van herplex simplex keratitisinfecties, polymorfismen in het humaan lactoferrinegen en de concentratie van dit eiwit in het traanvocht. Verder deed ze onderzoek voor het promotieproject van Nicolle Litjens. Ze keek hiervoor naar de effecten van fumaraten op de polarisatie van monocyt-verkregen dendritische cellen welke gestimuleerd waren met verschillende Toll-like receptor (TLR)-stimuli. Ook werd onderzocht of MMF, een specifiek type fumaraat, zijn effecten uitoefende via receptorbinding. Ten slotte werd onderzocht of er een associatie bestond tussen psoriasis en een IL-12B promoter polymorfisme. Tijdens deze stageperiode werd ook begonnen met het onderzoek naar madentherapie. Op 6 april 2004 behaalde Mariena haar doctoraal diploma *cum laude* en op 1 mei startte ze als AIO in dienst van de afdelingen Infectieziekten en Heelkunde van het Leids Universitair Medisch Centrum onder supervisie van Prof. Dr. Jaap T. van Dissel en Dr. Peter H. Nibbering. Initieel was het onderzoek gericht op de antimicrobiele activiteit van maden. Na ongeveer een jaar werd besloten om het onderzoek uittebreiden. Hiertoe werden de effecten van maden op processen betrokken bij de ontstekingsfase van wondgenezing bestudeerd. De bevindingen van het onderzoek naar de werkingsmechanismen van madentherapie zijn beschreven in dit proefschrift.

List of publications

van der Plas MJA, Andersen AS, Nazir S, Hensbergen PJ, van Tilburg NH, Oestergaard PR, Dohmen F, van Dissel JT, Bertina RM, Nibbering PH. Maggot secretions enhance plasminogen activator-induced fibrinolysis by cleavage of plasminogen. Manuscript in preparation.

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