Chapter 5

Maggot excretions inhibit
the human complement system

Maggot excretions inhibit complement

Submitted

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Abstract

The complement system plays an important role in the activation of the inflammatory response to injury, although inappropriate complement activation (CA) can lead to severe tissue damage. Maggot therapy is successfully used to treat infected wounds. In this study, we hypothesized that maggot excretions/secretions (ES) influence CA in order to modulate the host's inflammatory response. Therefore, the direct effect of ES on CA was investigated in healthy sera and in pre- and postoperatively gained sera from patients. Our results show that ES inhibited CA in human sera from healthy and postoperatively immune-activated human sera up to 99.9 %, via all three pathways of complement activation. ES proved to be temperature-tolerant and boiled ES showed superior complement-inhibiting properties. Protease treatment of ES reduced the complement-inhibiting effect markedly, indicating the involvement of an ES derived protein constituent in complement inhibition. This study indicates the first pathway independent complement-inhibitor that already is successfully used in clinical practice and may explain part of the improved wound healing caused by maggot therapy. Furthermore, the complement-inhibitor(s) present in maggot ES could provide a novel treatment modality for several diseases, resulting from an (over)active complement system.

Introduction

The complement system is part of the innate immune system and plays an important role in activation of the inflammatory response to injury.[1] However, inappropriate complement activation (CA) can also cause injury and can contribute to severe tissue damage, caused by the same mediators.[1,2] Three pathways of CA are recognized, viz. the classical pathway (CP), alternative pathway (AP) and mannose-binding lectin pathway (MBL-P).[1] Each pathway can specifically initiate the complement cascade, resulting in activation of the central factor C3 and finally reaching the terminal pathway of the complement system with factors C5b to C9. These latter factors form the membrane attack complexes (MAC).[1]

Maggot Debridement Therapy (MDT) is used to treat severely infected acute and chronic wounds.[3,4] This therapy was introduced in clinical practice in the 1930's by Prof. Baer, orthopaedic surgeon in Johns Hopkins Hospital in Baltimore, Maryland.[5] He showed that MDT healed osteomyelitis in children.[4] The discovery of penicillin by Fleming in 1928[6] supplanted MDT until its comeback in the 1980´s,[7] when antibiotic resistance became an increasing problem in resolving infections.[8]

Currently, MDT is widely used and shows beneficial effects in the healing of acute and chronic wound infections.[9,10] In 2004, MDT was approved by the US Food and Drug Administration (510[k] #33391).[11] One of the most leading hypotheses in literature is that maggots possess antibacterial activity,[12,13] however in our previous research, we found no direct antibacterial properties of maggots and/or their excretions/secretions (ES).[14] Definitively, MDT is effective in clinical practice.[9,10] Therefore, in this study, we searched for a possible indirect, immune-related activity of maggots to reveal their underlying mechanism of action in the process of wound healing.

We hypothesized that maggot ES could influence the innate immune system by affecting one or more of the three pathways of complement activation. The effect of ES on CA in healthy human sera was evaluated using different test methods for CA. Furthermore, the variation of the effect of ES on CA between different pools ES and between various donor sera was investigated, and the temperature tolerance of maggot ES was studied. Proteases and chelators were added to ES in order to evaluate the effect of ES on CA in donor sera after elimination of proteins and metal ions. At last, we investigated CA in pre- and postoperatively gained sera and thus tested the influence of ES in a clinically relevant situation.

Materials and methods

Collection of maggot ES

Sterile maggot ES of Instar-3 larvae (BioMonde GmbH, Barsbüttel, Germany) were collected according to the method as previously described.[14] Briefly, the maggots were incubated in sterile tubes for one hour at 35°C in the dark. Then, ES were removed by pipette and stored at -80°C. The protein concentration was determined using the Pierce Bicinchonic Acid Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA). Four different pools of ES with a concentration of 2000 μg/mL were used in this study.

Immunoassays

CA was investigated using the enzyme immunoassays from Wieslab™ (Euro-Diagnostica BV, Arnhem, the Netherlands) and the total hemolytic activity (CH50) assay. Every CA pathway was studied separately using the test methods from Wieslab™, developed and described by Seelen et al.,[15] and performed according to the manufacturer's instructions. Briefly, the individual CA tests constituted of a 96-well microtiter plate coated with specific activators for every pathway. The wells of the CP kit were coated with human IgM, those of the AP kit with lipopolysaccharide and those of the MBL-P-kit with mannan. Serum samples were obtained from four healthy individuals, directly aliquoted and stored at -80 °C. For the experiments, serum was diluted (CP and MBL-P 1:101, AP 1:18) in diluent containing specific blockers to certify that only the tested pathway was activated and 100 μL serum was added per well. MAC formation in the absence or presence of ES (up to 2000 μg/mL) was subsequently measured using a sandwich-ELISA principle.

The CH50 assay, performed as previously described by Kabat and Mayer,[16] measured the ability of a serum sample with and without presence of 2000 μg/mL boiled ES to lyse 50% of a standardized suspension of sheep erythrocytes (RIVM, Bilthoven, The Netherlands) coated with antibodies (Amboceptor, Behring, Amstelveen, The Netherlands). The degree of lysis is proportional with CA via CP and can be quantified by measuring the released hemoglobin at 415 nm.

In order to rule out any aspecific inhibitory effects of ES on a sandwich-based ELISA in general, a serum sample containing a known amount of IL-8 was re-analyzed in the presence of 1600 μg/mL boiled ES. For this purpose, IL-8 was measured using the Immulite 1000 platform (Siemens, Breda, The Netherlands), according to the manufacturer's instructions.

The effect of ES on CA in healthy sera

The effect on CA by ES was analyzed using four different healthy human donor sera (age range: 23-45 years) in the absence or presence of ES. A suboptimal

level of CA was used as a control in the absence of ES, in order to create a window for both complement inhibition and activation by ES. Therefore, a screening experiment was performed with diluted healthy sera in Phosphate Buffered Saline (PBS) to determine the dilution that showed a CA of about 50% compared to the optimal serum dilution as indicated by the manufacturer. The serum dilutions 1:202 for CP and 1:27 for AP and MBL-P were subsequently used and the resulting suboptimal CA was defined as 100%. A range of concentrations ES, generated by dilution in PBS was used: 2000, 1500, 1000, 500 and 250 µg/ mL.

Temperature tolerance of ES, protease sensitivity and chelator sensitivity
The stability of the complement-influencing properties of maggot ES was investigated under a range of different storage conditions. Therefore, aliquots of one pool ES were collected and stored at 4˚C or at room temperature. One aliquot from each was taken and tested after 1 day, 1 week and 1 month. Other aliquots were boiled for 15 minutes at respectively 60˚C and 100˚C before investigation. An aliquot containing fresh ES was used as the control in this experiment. The stability experiment was repeated with aliquots from ES boiled at 100˚C. In these experiments, only the CP and AP were tested for practical reasons.

Two different proteases were added to freshly collected ES, viz. proteinase K (Roche, Mannheim, Germany) and trypsin (Lonza, Verviers, Belgium), to degrade the proteins in ES. A final concentration of 20 µg/mL proteinase K or 25 µg/mL trypsin was added to a concentration range of ES. In another experiment, a concentration range of proteinase K and trypsin was added to 2000 µg/mL ES. The ES with proteinase K or trypsin was incubated for 15 minutes at respectively 72˚C and 37˚C, followed by 15 minutes at 100˚C in order to inactivate the added proteases before analyses of CA as previously described.

Chelators EDTA and EGTA (both from Merck, Darmstadt, Germany) were added to boiled ES in a final concentration of 0,25 mM to test the influence of sequestering divalent cations. Both chelators were also used as a control to show their potential effect without ES on CA.

Clinical pilot study with pre- and postoperatively gained sera
In this clinical pilot study, CA was investigated using sera that were gained pre- and postoperatively from trauma patients in our clinic, who were scheduled for an operative procedure (6 patients for an osteosynthesis, 1 patient for major removal of metal hardware). The experiments were performed with serum samples from seven patients. All patients had a monofocal fracture of an extremity that was operated at least 7 days after the initial trauma. Excluded were patients with auto-immune diseases, the use of immunosuppressive drugs, malignancies, liver function disorders and/or the use of more than 4 units alcohol a day. Samples

were obtained before the operation, 6 hours after surgery and 24 hours after surgery and the CA was determined for every sample with and without addition of maggot ES.

Percentage of CA
The percentage of CA was calculated, after subtracting all OD-values measured at 620 nm from those measured at 405 nm (ΔOD), using this formula:
(ΔOD (sample) - ΔOD (negative control)) /
(ΔOD (suboptimal positive control) - ΔOD (negative control)) x 100%

Statistics
All experiments were performed in quadruplicate at least. The Student's t-test for independent variables was used to analyze the influence of ES on CA. The mean CA of each sample with ES was compared with the mean CA of the positive control of that specific experiment. To compare the mean CA in the pre- and postoperatively gained sera among the three different times, the ANOVA repeated measures was used and followed by the Bonferroni correction. The variation between the sera and the different pools ES was analyzed by one-way ANOVA, followed by the least significance difference (LSD) post hoc test. All statistical analyses were performed using SPSS® for Windows®, Version 14.0 (SPSS Inc, Chicago, IL). Values of $p < 0.05$ were considered significant.

Results

Direct and indirect effect of ES on CA
Maggot ES in the absence of serum did not show any CA for the three individual pathways in the Wieslab™ system. All samples displayed 0% CA (all p<0.0001; data not shown). In order to examine the effect of ES on CA in healthy donor sera, a serum dilution was used displaying suboptimal CA in the absence of ES (~50% of the optimal level, subsequently set to 100%) (Fig. 1), creating a window for both enhanced and decreased CA in the presence of ES. Enhanced CA by ES was not observed in any of the tested healthy donor sera. In contrast, CA in all healthy donor sera was clearly reduced by maggot ES via all pathways analyzed. The highest protein concentration of ES showed the strongest complement inhibition and the concentration of ES correlated logarithmically with the effect on CA. The CA was reduced to a minimum of 4,4 % for CP (p<0.0001), to 18,8 % for AP (p<0.0001) and to 5,7 % for MBL-P (p<0.0001) (Fig. 2a). The AP could be further inhibited to 7,4% by addition of more ES proteins to the serum (p<0.0001; Fig. 2b).
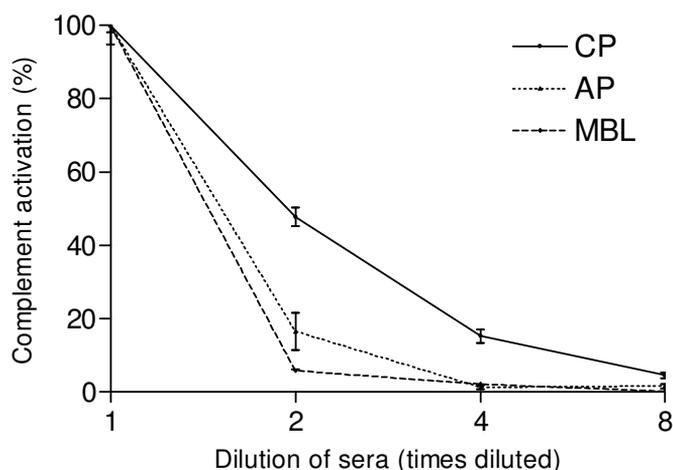
Figure 1 CA of diluted donor sera

CP have an optimal dilution of 1:101, which was defined as 100% and AP and MBL-P have an optimal dilution of 1:18, which was defined as 100%. On the x-axis 2, 4 and 8 correspond with a dilution of 1:202, 1:404 and 1:808 for CP and with a dilution of 1:36, 1:72 and 1:144 for AP and MBL-P. The serum dilutions 1:202 for CP and 1:27 for AP and MBL-P correspond with 50% of CA and were used as suboptimal controls of CA in further experiments.

The inhibition by ES of CA was independent of the healthy donor serum used; no significant variation was observed for the CP (p=0.120), AP (p=0.079) and MBL-P (p=0.382) (Fig. 3a). In addition, neither varied the effect of different ES pools on one healthy donor serum for CP (p=0.115) and MBL-P (p=0.554), however it did vary to some extent for AP (p=0.002) (Fig. 3b). To rule out any aspecific inhibitory effects of ES on a sandwich-based ELISA in general, ES was added to a serum sample containing a known amount of IL-8. Subsequent re-analysis of IL-8 showed an almost identical IL-8 concentration (data not shown).
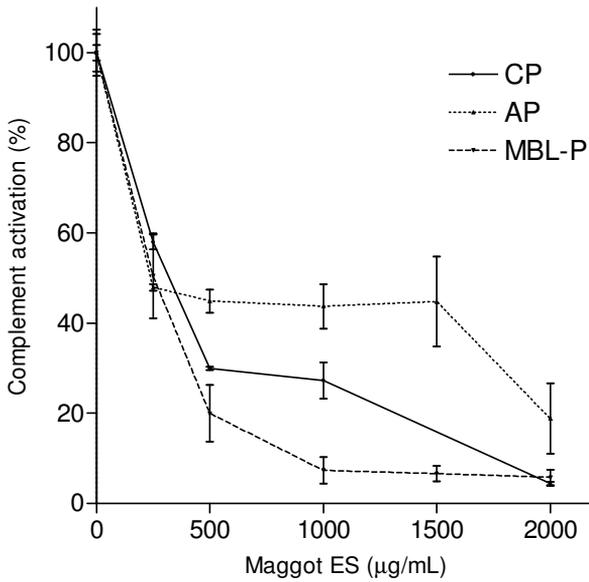
Figure 2A The effect of ES on CA in healthy sera
The results showed a logarithmically correlation between the protein concentration of ES and their potency to inhibit complement. CA was reduced to a minimum of 4,4% for CP (p<0.0001), to 18,8 % for AP (p<0.0001) and to 5,7 % for MBL-P (p<0.0001).

In order to confirm the results described above, a different system for the analysis of CA via the CP was used, the total hemolytic activity (CH50) assay. In the absence of ES a healthy donor serum showed 98% CA. In contrast, in the presence of the highest ES concentration a reduction of CA of 39% was observed (p<0.0001; data not shown).
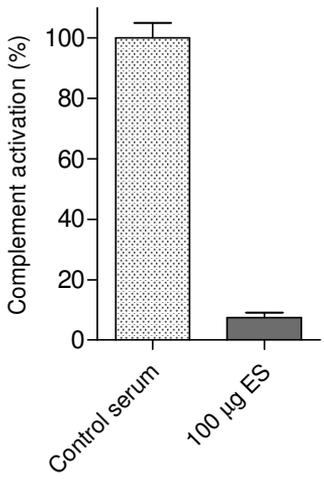

Figure 2B The influence of a high protein concentration of ES (100 µg/well) on CA via the AP. The addition of more ES proteins (50 µl of 2000 µg ES/mL) to the sera resulted in stronger complement reduction with a minimum of 7,4 % (p<0.0001).

Heating of ES improves their complement inhibiting properties
All ES samples reduced CA comparable with the control, irrespective of storage time or temperature (CP: all p-values ≤0.0003; Fig. 4a, AP: all p-values ≤0.0095; data not shown). The complement reducing ability of ES boiled at 100˚C was 97,8% for CP and 88,3% for AP, thus appeared even higher than fresh ES (CP: 86,8%; boiled ES versus fresh ES p=0.0001, AP: 49,6%; boiled ES versus fresh ES p=0.0024). ES boiled at 100˚C and then stored at room temperature for a month showed highly conserved complement inhibiting properties: 95,4% inhibition for CP (p<0.0001; Fig. 4b) and 99,9% for AP (p<0.0001; data not shown).

Degradation of proteins in ES partially reduces their complement inhibiting effect
Boiled ES in presence of the highest concentration of proteinase K or trypsin showed respectively 35% and 32% less complement inhibition (for both ES/ proteases versus control ES: all p-values <0.0001; Fig. 5) compared to ES without proteases. This effect was similar for all tested ES concentrations (data not shown).

Effect of chelators on complement inhibition by ES
Controls of CA in the presence of chelators EDTA and EGTA already showed inhibition of CA, respectively 88% and 90% (both p-values <0.0001; Fig. 6). As result, the effect of chelators on ES activity could not be determined in the current experimental setup.
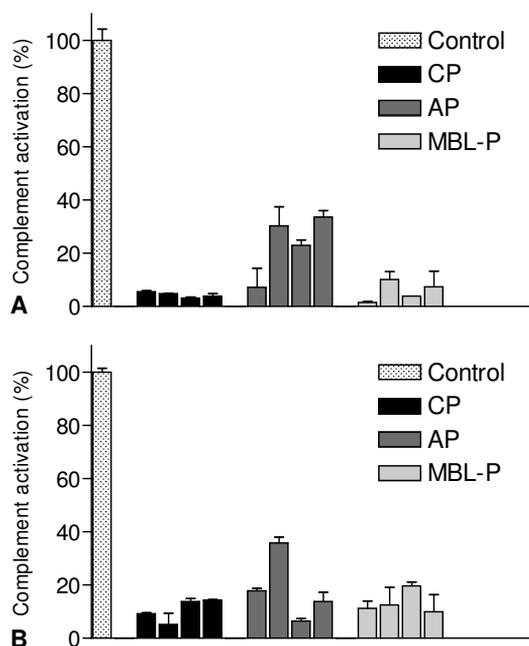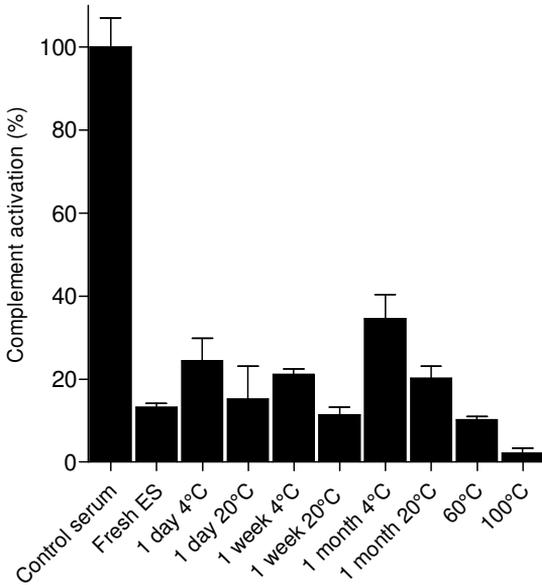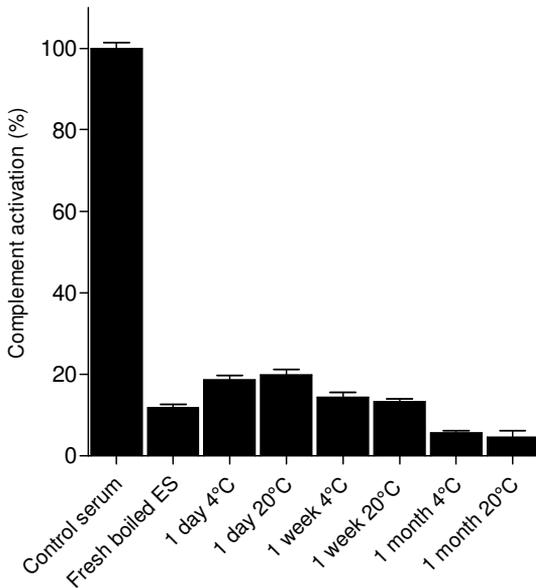

Figure 3 A-B The variation of the effect of ES on CA between a) four different healthy donor sera and b) different pools ES

The influence of ES (2000 µg/mL) on CA between the donor sera did not vary for the CP (p=0.120), AP (p=0.079) and MBL-P (p=0.382), neither differed the effect of different ES on one healthy donor serum for CP (p=0.115) and MBL-P (p=0.554), however it did vary for AP (p=0.002).

A

B

Figure 4A-B Temperature tolerance of a) freshly collected ES and b) boiled ES, tested on the CP

All stored ES reduced CA compared with the control. ES stored at room temperature for a month still showed 79,7% inhibition (p<0.0001). Boiled ES at 100˚C had the best complement-inhibiting properties and reduced 89% to 98% of the CA (all p-values <0.0001). Storage of boiled ES at room temperature for a month did not decrease their complement-inhibiting capacity. The experiments for the AP showed similar results comparing to the CP.
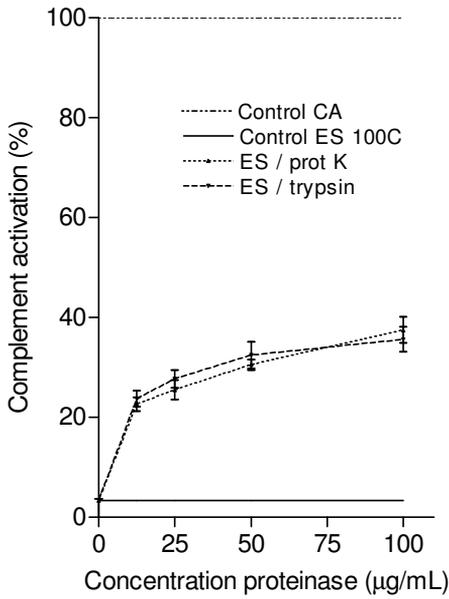
Figure 5 Protease sensitivity of the complement inhibiting properties of ES tested on the CP
ES in presence of proteinase K or trypsin showed respectively 35% and 32% less complement inhibition compared to control ES (both p-values <0.0001).
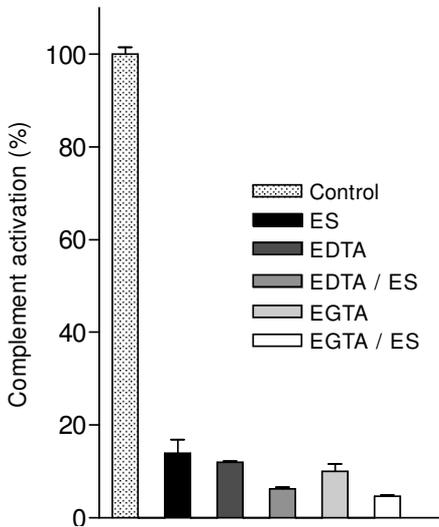

Figure 6 Chelator sensitivity of the complement inhibiting properties of ES tested on the CP
Both chelators inhibited CA themselves, until 88% for EDTA (p<0.0001) and 90% for EGTA (p<0.0001), and did not differ the effects of ES on CA.

The effect of ES on complement activation in post-operative serum

The mean level of CA in the first 24 hours after surgery compared to the pre-operative control CA was 98% for the CP (p=0.757; Fig. 7a), 102% for the AP (p=0.153; Fig. 7b) and 115% for the MBL-P (p=0.530; Fig. 7c). In presence of ES the CA via CP was reduced to minima of 19 up to 25% (all p-values <0.0001; Fig. 7a), via AP reduced to 43 up to 55% (all p-values<0.0001; Fig. 7b) and via MBL-P reduced to 15 up to 19% (all p-values <0.0001; Fig. 7c). These results clearly indicate that maggot ES also inhibits complement activation in a clinically relevant patient population, those eligible for MDT. Table I shows an overview of the individual results of all patients included in the study.

Discussion

In this study, we investigated the influence of maggot ES on CA in healthy and post-operatively gained donor sera. The principal finding was that all tested ES inhibited CA in healthy and immune-activated sera. Boiled ES had the best complement inhibiting properties and reduced CA until a maximum of 99,9%. We found a logarithmical correlation between the protein concentrations of ES and their complement-inhibiting effect. These results may explain part of the improved wound healing that is observed when MDT is used in clinical practice[3,4,9,10,17] and could provide new insights into the role of CA in chronic wounds.

At first sight, the complement-inhibiting effect of ES may seem contrary as MDT heals severe, infected wounds. Therefore, it could be expected that a stimulation of CA is needed to clear the debris, destroy pathogens and overcome the inflammatory phase of wound healing. However, it is noted that inappropriate CA can cause injury and contribute to severe tissue destruction.[1] Probably, the CA in patients that suffer from acute and chronic wound infections is hampering the wound healing more instead of stimulating it.[2]

Evolutionary, maggots need to have a strategy to evade the activation of the complement system.[18] In nature, larvae infestate the skin of some animals to feed themselves with internal tissues.[19] Their colonization causes myiasis in the affected animal, which can be fatal when untreated.[19] Veterinary medical literature already suggested that maggots could have immunosuppressive effects in sheep,[20] and larvae of the *Anasakis simplex*, a parasitic worm, previously showed to have complement-inhibiting properties.[21] In human-beings the *Lucilia sericata* maggots, which are used for MDT, colonize wounds, but cannot cause harmful myiasis, because they limit their actions to necrotic tissues and spare the healthy tissues for unknown reasons. Humans do not develop an inflammatory and/or immune reaction against the maggots, which can be explained by the results in this research: the larvae are able to suppress our complement system.

Table 1 An overview of the CA of the pre- and postoperatively gained sera with or without the addition of maggot ES

Patient Number	Sexe (M/F)	Age (years)	Fracture	Surgery	Pre-operative CA (%) CA / CA with ES	Postoperative CA 1 6 hours (%) CA / CA with ES	Postoperative CA 2 24 hours (%) CA / CA with ES
1	M	28	Bimalleolar	Screws	CP: 100% / 29% AP: 100% / 28% MBL-P: 100% / 21%	108% / 33% 126% / 74% 107% / 36%	109% / 38% 112% / 65% 103% / 23%
2	M	26	Humerus	LCP[1]	CP: 100% / 27% AP: 100% / 58% MBL-P: 100% / 13%	103% / 19% 101% / 47% 104% / 7%	105% / 24% 108% / 85% 107% / 21%
3	V	72	5th Metacarpal	Plate	CP: 100% / 28% AP: 100% / 81% MBL-P: 100% / 16%	99% / 25% 95% / 71% 92% / 6%	x x x
4	M	50	Distal radius	Plate	CP: 100% / 31% AP: 100% / 78% MBL-P: 100% / 11%	101% / 32% 88% / 28% 85% / 8%	x x x
5	M	65	Humerus	LCP[1]	CP: 100% / 31% AP: 100% / 68% MBL-P: 100% / 5%	98% / 25% 99% / 27% 122% / 7%	97% / 19% 97% / 81% 170% / 3% [*]
6	M	40	Calcaneus	Plate	CP: 100% / 20% AP: 100% / 62% MBL-P: 100% / 48%	97% / 14% 113% / 58% 93% / 17%	96% / 13% 103% / 24% 99% / 33%
7	V	29	Femur	Removal Intramedullary nail	CP: 100% / 16% AP: 100% / 13% MBL-P: 100% / 24%	95% / 11% 63% / 4% 101% / 26%	82% / 8% 95% / 19% 99% / 17%

LCP = locked compression plate
x = patient was already dismissed within 24 hours
[*] = initially very low complement activation via MBL-pathway

The use of boiled ES resulted in higher complement-reducing capacity than the use of directly collected ES. Superior results were found when boiled ES was stored at room temperature for one month. So, ES are very thermo-stabile and heating improves their complement-reducing properties. Furthermore, we found that proteases decrease the complement inhibition of ES. Possibly, the substance in ES that inhibits each of the three pathways of CA is a boiling stable protein that influences a central point of the complement cascade, e.g. C3 or one of the factors that form the MAC's. This hypothesis will be investigated in further

research.

The results in this in vitro study could differ from those in vivo. However, the conditions of the experiments were adjusted as much as possible to the clinical situation. For example, maggots were incubated in darkness at 35°C and all ES had an acidity of pH=8. Moreover, different clinically applied test methods were performed with consistent results. Furthermore, CA by CP and MBL-P may seem more reduced than CA by AP, but the results between these cannot be compared equally, because it was necessary to maintain the optimal serum dilutions and therefore we could not similarly perform the experiments.

This is the first study that examined and showed in vitro complement inhibition by maggot ES. Previous research into the underlying modes of action of MDT showed inhibitory effects of ES on biofilm formation[22] and on multiple neutrophil pro-inflammatory responses[23] and have described the beneficial effect of maggot ES on the modulation of extracellular matrix components by which they could enhance tissue formation and accelerate healing.[24,25] Live maggots or ES do not posses direct antibacterial activity,[14] so in an infected wound, either there is another mechanism that leads to the reduction of the bacterial growth or the wound is able to heal despite bacterial colonization. As a result of complement inhibition by ES, the inflammatory response is also suppressed. Perhaps the bacterial colonization itself does not impair wound healing, but only the prolonged inflammatory response to the bacteria, which characterizes chronic wounds, is harmful.

In recent literature, the only known pathway independent complement inhibitor that may be used for the treatment of complement-mediated diseases in clinical practice like ES, is Compstatin, isolated from a phage-displayed random peptide library.[26] One of the leading concerns of the clinical use of non-specific complement inhibitors is that they could have adverse consequences for patients, such as (recurrent) infections.[1,18] However, MDT (and thus ES) already is in clinical use for many years without any described side-effects in literature, nor in our own clinical experience over the last ten years.[4,9]

If the substance that is responsible for the complement inhibition can be isolated, this could provide a new treatment for all diseases, resulting from an (over)active complement system. In future research, we will examine the possible therapeutic applications of maggot ES as a pathway independent complement inhibitor.

Acknowledgement

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