Down-regulation of antibacterial peptide synthesis in an insect model induced by the body-surface of an entomoparasite (*Steinernema feltiae*)

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Abstract

This study focuses on the interaction between an entomopathogenic nematode and an insect model to further investigate the role of parasite body-surface and its compounds (particularly epicuticular lipids) in the immunosuppression of host defences. Our goal was to ascertain the potential interference of the parasite epicuticular lipids with the antimicrobial response mounted by Gram-negative bacteria-challenged hosts. Since the parasite model used in this study (*Steinernema feltiae*) releases symbiontic bacteria in the host hemocoel during the late phase of infection, the inhibition of the antimicrobial response could be needed to avoid the activity of host factors potentially harmful for the microorganisms.

After bacterial challenge, when insect hosts were infected with purified parasite cuticles, we always observed lack of bacterial clearance concurrently with the absence of hemolymph low molecular weight components. The observed effects seem to be related to the interaction of parasite cuticular lipids (PCLs) with specific components of the host hemolymph; these host interacting proteins (HIP17, HIP26 and HIP35) were removed by the parasite, and their absence (or reduction) apparently prevented antimicrobial peptide synthesis. The inhibitory properties were lost when cuticles were pre-treated with compounds (such as lipase or methanol-chloroform) affecting their lipidic moiety. Moreover, the key role of epicuticular lipids was also confirmed by the inhibitory properties of methanol-chloroform extracted lipids, which were comparable to those of parasite whole cuticles.

Finally, the involvement of HIPs was assessed by their partial purification followed by injection into the host. When HIPs were co-injected with bacteria into cuticle-inhibited larvae, the antimicrobial activity was completely restored.

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1. Introduction

Antibacterial peptides in insects represent the main inducible humoral defence factors against bacterial infection [1–3]. Physiologically, the presence of microorganisms in the hemocoelic cavity triggers various immune processes such as proPO system activation, cell-mediated nodulation and phagocytosis [4–6]; however, these processes are effective only in the presence of a limited infection. When bacterial proliferation eludes short-term first line defences, the antibacterial response is the most effective immunological mechanism available to the host to prevent septicaemia.

Activation of insect immune genes [7,8] is considered as a humoral response homologous to vertebrate innate immunity, since this process is mediated by phylogenetically conserved Toll/IMD pathways [9,10]. Vertebrates and invertebrates share either
recognition steps, consisting in specific interactions between pathogen associated molecular patterns (PAMPs) and pattern-recognition receptors (PRRs) [11,12], and intracellular signal transduction cascades leading to the synthesis of antibacterial/antifungal peptides [13].

Innate immune recognition is based on the detection of structural and conserved products of microorganisms of a given class. This cost-effective recognition process allows a limited number of germ-line-encoded PRRs to recognize any microbial infection. Thus, recognition of the conserved lipid-A pattern in LPS, for example, allows a single PRR to detect the presence of almost any Gram-negative bacterial infection. Furthermore, PAMPs are essential for microbial survival, as mutations or loss of PAMPs are often lethal for microorganisms. The presence and recognition activity of hemolymph PRRs are essential to trigger effector mechanisms of innate immunity. Any interference with the PAMPs–PRRs recognition process can lead to the loss of effector responses impairing the host’s ability to eliminate foreign invaders. The above described strategy is exhibited by entomopathogenic nematodes that by interacting with insect host hemolymph humoral factors, either prevent proPO system activation or elude cellular encapsulation [14].

Entomopathogenic nematodes belonging to the genera *Steinernema* and *Heterorhabditis* carry bacterial symbionts, *Xenorhabdus* sp. and *Photorhabdus* sp., respectively, in the gut of the infective juvenile stage. Parasites actively penetrate into the haemocoel of insect hosts; after 1–2 h from penetration, the nematodes unleash the symbiotic bacteria and within 24–48 h the host succumbs to bacterial septicemia [15].

Suppression of immune reactions is one of the main mechanisms governing the outcome of relations between a potential parasite and its host(s) [16–18]; in the entomopatho-parasite–insect model the success of the deadly advanced stage of the infection is strictly dependent on the immunodepressive activity performed by the parasite itself early after penetration of the host. Generally, parasites have developed two main strategies to escape ‘non-self’ recognition: immunoevasion and/or immunodepression achieved either by active release of toxic compounds or removal of host humoral factors [19,20].

Parasites may successfully colonize their host by evading recognition and thus preventing activation of effectors mechanisms; circumvention of the host immune system can be achieved by molecular mimicry (or disguise) strategies, or colonization of young hosts or host tissues with low immunocompetence; alternatively (or concurrently) many parasites are able to depress either cell-mediated or humoral effectors mechanisms. Active evasion strategies are commonly referred to as interference [21]; in this case, parasites show an aggressive suppression or alteration of the host immune system defences. Interference can be directed toward host humoral factors that are neutralized by the parasite, or immunocompetent cells could be targeted [22].

With regard to humoral depression, proPO system is one of the main targets for many parasites; this is probably due to the need to neutralize its drastic and rapid effects when a host is infected. In a previous paper we observed a drastic inhibition of phenoloxidase activity in the hemolymph of *G. mellonella* induced by living and heat-killed entomopathogenic nematodes (*S. feltiae*); surprisingly, the same inhibitory effects were observed also following the injection of isolated and purified parasite cuticles [23].

In parasites, besides the function of protection from the environment, the body-surface (cuticle and/or epicuticle) also mediates interactions with the host. Thus, these immunoevasion strategies often involve the parasite body surface. Parasites’ success may arise partly from the ability to directly modulate host immunity, partly from their cuticle’s (or other body envelope’s) capacity to circumvent cellular and humoral defenses of the host [19].

The role of parasite cuticular lipids (PCLs) in the interaction and removal of putative PRRs (arbitrarily named host interacting proteins; HIPs) from host hemolymph has been discussed in a recent review [24]. The specificity of the above interactions could be due to a structural correlation of cuticle lipids to bacterial LPS [14] and this mechanism might be responsible for the lack of host humoral and cellular defences. In addition, the affinity of the parasite body-surface molecules for host hemolymph components could result in a coating of the nematode consisting of host self-proteins, thus, producing a molecular disguise process against cellular encapsulation [14,24].

In the current work we have focused on the antibacterial response of *Galleria mellonella* (host model) after experimental injection with isolated parasite cuticles. Our data demonstrate an additional role of the parasite body-surface, since its interference is directed also against host antibacterial peptide synthesis. Moreover, the inhibitory features of the parasite cuticle could be explained by its binding specificity, which results in the removal of some
humoral hemolymph components (HIPs) normally involved in host immunity.

2. Materials and methods

2.1. Chemicals

All reagents were of the highest available grade and were purchased from: Boehringer Mannheim GmbH (Pentzberg, Germany); ICN (ICN Biomedicals, GmbH, EU); Biorad Laboratories (Richmond, CA, USA); Sigma Chemicals (St Louis, MO, USA) and Difco Laboratories (Detroit, MI, USA). Laboratory equipment was from BioRad, Sorvall (Dupont, Newtown, CT, USA).

2.2. Biological species

Galleria mellonella (Lepidoptera, Pyralidae) larvae were reared on a sterile mixture of food (Brivio et al., 2002). Only healthy late stage caterpillars were selected for the experiments.

Steinernema feltiae (UK strain) were provided by Bioplanet (Cesena, Italy). Nematodes were purified from the commercially available product NemoPAK-S®.

Briefly, nematodes were separated from the inert carrier material by several washes with tap water, then centrifuged in a Sorvall swing-out rotor at 1000\( \times \)g for 10 min at 20\( ^\circ \)C on a discontinuous (25%–50–75%w/v) saccharose gradient. Clarified parasites were collected at the interface between 25 and 50% of the saccharose gradient tube and washed several times with tap water and centrifuged at 1800\( \times \)g for 10 min at 20\( ^\circ \)C. Gram-negative (Enterobacter cloacae and Escherichia coli) bacteria were cultured overnight at 37\( ^\circ \)C in 20% LB Broth (DIFCO). Bacterial concentration was estimated spectrophotometrically by absorbance values (\( \lambda = 540 \) nm).

2.3. Parasite cuticles: isolation and treatments.

Purified S. feltiae cuticles were obtained from parasites isolated as described above.

Washed nematodes were suspended in 20 volumes of CEB (cuticle extraction buffer: 10 mM Tris–HCl, 10 mM EDTA, 1 mM PMSF, pH 7.2) and sonicated with two 150-watt bursts for 3 min in a Labasonic-L Ultrasonic processor (B. Braun Biotech, Inc., Allentown, PA, USA). Parasite body fragments were then homogenized in a glass homogenizer (Potter Dounce, B. Braun, pestel B), with 10–15 strokes, to remove internal tissue debris and body fluids, finally, separated cuticles were extensively washed in sterile Tris buffer (10 mM, pH 7.2). The degree of purification was checked under a stereo-microscope and finally assessed by light microscopy.

Isolated cuticle fragments, re-suspended in Tris buffer, were injected into insect hosts; alternatively, cuticles were pre-treated with lipase (5 U ml\(^{-1}\) lipase in 10 mM Tris–HCl, pH 7.2) for 60 min at 37\( ^\circ \)C, or with methanol–chloroform (1:2, v/v), for 60 min at room temperature. Moreover, injection assays were performed with lipidic extracts obtained after methanol–chloroform treatments; solvents were removed by overnight evaporation under a nitrogen stream and pellets of cuticular lipids resuspended in Tris buffer before the injection.

2.4. Bacterial infection of G. mellonella (in vivo bacterial growth)

Insect larvae were surface-sterilized with ethanol (70%), bacteria (nalidixic acid-resistant E. cloacae strain) were resuspended in sterile Tris buffer at a concentration of 2\( \times \)10\(^6\) bacteria ml\(^{-1}\). 10\( \mu l \) of bacterial suspension was injected into the hemocoel cavity of G. mellonella larvae using a gas-tight syringe (Hamilton Co., Reno, NE, USA) with 0.21 mm (internal diameter) needles. As controls, insects were injected with 10\( \mu l \) of sterile Tris buffer or Tris buffer plus cuticle fragments.

To ascertain if nematode surface (cuticles) were able to inactivate or reduce antibacterial response of G. mellonella, double injection assays were performed. Parasites cuticles, prepared as described above, were resuspended in sterile buffer (10 mM Tris HCl, pH 7.2). Approximately 15–20 cuticle fragments (about 10\( \mu l \) of purified cuticle suspension) were injected into the hemocoel cavity of G. mellonella larvae. Thirty minutes after the first injection, larvae were infected with bacteria as described above. The same experiments were also performed with lipase, methanol–chloroform pre-treated cuticles and methanol–chloroform lipidic extracts.

In all experiments larvae were bled 2, 4, 8 or 24 h after the injection. Hemolymph was obtained from punctured prolegs of the last instar larvae; whole hemolymph samples were then processed by two low-speed centrifugations (700\( \times \)g for 10 min, at 4\( ^\circ \)C) to remove cells and tissue debris.

Aliquots of cell-free fractions (CFF) supernatants were plated on Petri dishes (20% LB, 15% agar, 25\( \mu g \) ml\(^{-1}\) nalidixic acid) or analysed by 16%
acrylamide Schagger-PAGE [25] revealed by silver staining. Petri dishes were incubated overnight at 37 °C, bacteria were quantified by Colony Forming Units (CFU) count.

In addition, bacterial growth in host hemocoel cavity was assayed visually by light microscopy in microwells cultures of whole hemolymph diluted in insect Grace medium (Sigma).

2.5. In vitro hemolymph antibacterial activity assays

CFF antibacterial activity was assayed by in vitro analysis on streptomycin-resistant E. coli strain. Briefly, 10 µl CFF of infected larvae were added to 1 ml of bacterial suspension (2×10^4 E. coli ml⁻¹ in sterile LB). After 1 h at 37 °C bacteria were pelleted (at 4500×g for 10 min) and resuspended in 1 ml of LB. 100 µl of suspension were plated (20% LB, 15% agar, 100 µg ml⁻¹ streptomycin). Petri dishes were incubated overnight at 37 °C. Bacteria were quantified by CFU count. As controls, antibacterial activity assays were performed adding CFF of naı¨ ve larvae to the bacterial suspension. To test hemolymph low and high molecular weights factors, all experiments were carried out with CFF previously ultrafiltered by Microcon™ (Millipore Co.) YM-10 centrifugal filters (cut-off 10 kD).

2.6. Effects of HIPs (host interacting proteins) on antibacterial activity.

As mentioned, we identified G. mellonella hemolymphatic proteins sequestered by S. feltiae body-surface; these proteins (HIPs) were assayed as enhancer of host antibacterial induction pathways. A first injection with cuticles was performed as described above; a second injection was carried out with 0.5 or 1 µg of isolated HIPs (HIPs partial purification is schematically described in Fig. 5) in 10 µl of E. cloacae suspension (2×10^6 bacteria ml⁻¹). After bleeding, CFF samples were plated as described above or analysed by electrophoresis (Schagger-PAGE); Petri dishes were incubated overnight at 37 °C and bacteria quantified by CFU count.

2.7. Data processing and statistical analysis

Differences between mean values were evaluated using the Student’s paired t-test and considered significant when P<0.05. All experiments were replicated at least five times. Data were processed with KaleidaGraph™ 3.51.

3. Results

3.1. Effects of nematode body-surface (cuticle) on host antibacterial activity

We carried out experimental bacterial infection of G. mellonella last instar larvae with the aim to confirm the ability of the host to mount antimicrobial response against Gram negative bacteria. In addition, we assayed the antimicrobial activity in both low and high molecular weight fractions obtained by fractionating cell-free hemolymph samples. As shows Fig. 1 (in vitro antibacterial assay), 12 h after infection with Gram-negative bacteria, G. mellonella hemolymph low molecular weight fraction (Fig. 1, graph, e<10) shows a strong antimicrobial activity and the occurrence of newly synthesized peptides (Fig. 1, right panel, inset, arrowheads). We considered CFU confluence as corresponding to a 100% in vitro bacterial count.

![Fig. 1.](image-url) In vitro antibacterial activity of G. mellonella larvae. Left: a graph showing antimicrobial activity in fractionated host hemolymph. After E. cloacae injection larvae hemolymph cell-free fraction was collected and fractionated with microcon filters (cut-off 30 kD). Separated fractions were incubated with E. coli cultures for 1 h at RT and aliquots cultured on Petri dishes. CFU counts were used to evaluate bacterial killing properties of hemolymph fractions. Strong antimicrobial activity was observed in the low molecular weight fraction (e<10) from bacteria-challenged larvae. B: bacteria culture; e>10 and e<10: high and low fractions of hemolymph from naïve larvae; e>10 and e<10: high and low fractions of hemolymph from bacteria-injected larvae. Right, tricine-PAGE showing the appearance of antimicrobial peptides (arrowheads) in the hemolymph of bacteria-challenged larvae (lane B); lane A: electrophoretic pattern of samples from naïve larvae.
We had previously observed a drastic effect on antibacterial activity in the hemolymph of *G. mellonella* after the injection of whole nematocomplexes; in order to exclude the involvement of nematode secretions and/or released bacteria, we carried out all the injection assays with isolated parasite cuticles. Cuticle fragments, purified as described in Section 2, were injected into the hemocoel cavity of host last instar larvae; after 30 min larvae were infected with $2 \times 10^4$ *Enterobacter cloacae*, at several times namely 2, 4, 8, and 24 h after the infection larvae were bled and hemolymph antibacterial activity was tested by bacterial count. In Fig 2, the graph shows a marked inhibition of antimicrobial activity due to the presence of parasite cuticles (Fig. 2, CB); as expected, when the first injection (cuticles) was omitted, within 8 hours after bacterial infection a complete clearance was observed (Fig. 2, B). No bacterial growth was evident in control assays (Fig. 2, T, and CT).

### 3.2. Cuticle treatments affect its inhibitory properties

To confirm the hypothesis of an active role of lipidic compounds of the parasite epicuticle, we assayed the inhibitory role of the lipidic moiety of the parasite body-surface by injection assays with chemically (methanol–chloroform treatments) and enzymatically (lipase)-treated cuticles. The data obtained clearly showed that both cuticle treatments caused a drastic loss of inhibition of the host antibacterial activity (Fig. 3, C$_{MCB}$, C$_L$B).

We also tested the effects of isolated lipidic compounds; after the injection of whole cuticular lipidic extracts, obtained after methanol-chloroform treatments, no antibacterial activity was observed (Fig. 4, E$_{MCB}$). Furthermore, to exclude that the observed inhibitory effect was not due to extraction solvents, methanol-chloroform buffer was injected as a control (Fig. 4, MCB).

### 3.3. Host hemolymph components (HIPs) restore antimicrobial activity.

Three main proteins, the Host Interacting Proteins (HIPs), are sequestered by the parasite after entry into the *G. mellonella* hemolymph. In order to partially purify them, we carried out interaction assays and high salt elution, applying the method described in Fig. 5. For the purpose of assessing the role of host hemolymph components removed by the parasite, we...
performed modified double injection assays: host larvae were previously injected with parasite cuticle fragments, then with *E. coli* plus 0.5–1 mg ml⁻¹ of partially purified HIPs. After the elution, HIPs were co-injected with bacteria and time-course of hemolymph clearance evaluated by bacterial count (Fig. 6). As shown in Fig 6, C-HIPsB, the addition of the parasite-sequestered components resulted in a marked recovery of hemolymph antimicrobial activity, moreover the observed effects are seemingly dose-dependent, since increasing the concentration of added HIPs resulted in a decrease in the inhibition of antibacterial activity due to parasite cuticles (Fig. 6, C-HIPsB 0.5 µg ml⁻¹, C-HIPsB 1 µg ml⁻¹).

Significant data obtained by the assays are summarized and visualized by histogram in Fig. 7.

3.4. Antibacterial peptides in hemolymph of treated/untreated larvae

To evaluate the presence or absence of low molecular weigh compounds with antimicrobial activity, analytical electrophoresis (Tricine-PAGE) was performed. All the analyzed samples were from hemolymph of larvae infected with *E. cloacae*, previously injected with: purified cuticles, cuticles plus HIPs, lipase-treated cuticles, methanol–chloroform-treated cuticles and extracted lipidic compounds; as a control samples from Tris-injected larvae were tested (Fig. 8, T). In all assays larvae were bled 24 h after the bacterial infection.
Both the cuticle-injected (Fig. 8, CB) and cuticle lipidic extract-injected (Fig. 8, E\textsubscript{MC}B) samples show the absence of small peptides reflecting the inhibitory properties of the parasite body-surface components. When sequestered hemolymph molecules (HIPs) were co-injected with bacteria, the antimicrobial activity of the larvae was restored; as showed in Fig. 8, lane C-HIPsB, two bands of about 9 and 4 kD are clearly observable. Similar patterns were observed when cuticles were pre-treated to modify or remove lipids; AMPs synthesis was not inhibited either with lipase (Fig. 8, C\textsubscript{L}B) or methanol–chloroform (Fig. 8, C\textsubscript{MC}B) cuticle treatments. Moreover, lipidic compounds extracted from parasite cuticles seem to be able to suppress antimicrobial production (Fig. 8, E\textsubscript{MC}B).

3.5. Bacterial growth in cultures of host hemocytes

In order to assess the presence of bacterial cells in the host hemolymph, we carried out short-term in vitro cultures in microwells with whole host hemolymph from \textit{E. cloacae}-injected larvae (Fig. 9, A).

Twenty minutes after culture, no bacterial cells are detectable among round-shaped hemocytes (not adherent), instead, in Fig. 9, panel B, due to the presence of injected cuticles, a significant number of bacteria is noticeable as a result of the inhibitory effects of the parasite body-surface on antibacterial peptide synthesis; moreover, the hemocyte population is markedly reduced probably due to cell damage induced by released bacterial products. In the same figure, panel C shows restoration of bacterial clearance (no bacteria observed), as injected parasite cuticles were previously treated with lipase to remove their inhibitory properties. Moreover, the micrograph shows the cell-mediated encapsulation process of cuticle fragments (arrowheads). In all the assays \textit{G. mellonella} larvae were bled 12 h following injection.

The graph (at the bottom of Fig. 9) summarizes total bacterial counts (bacterial cells 100 \textmu l\textsuperscript{-1}) in the hemolymph of larvae from the assays related to the above micrographs. Both in lipase-treated (C\textsubscript{L}B) and bacteria-injected (B) host larvae assays, bacterial cells were absent; instead, up to 1.3×10\textsuperscript{3} \textmu l\textsuperscript{-1} bacterial cells were estimated in hemolymph samples from larvae injected with untreated cuticles (CB).

4. Discussion

Insect immune responses resemble those in vertebrate innate immunity [26] and involve both cellular and humoral defense mechanisms that are triggered by PRRs capable of specific binding to PAMPs [27]. PRRs can mediate ‘non self’ killing directly, through phagocytosis, or indirectly, by the triggering of serine proteases that in turn can activate defense reactions such as melanotic encapsulation [28–30] or initiate intracellular immune signalling pathways which regulate the transcription of antimicrobial peptide genes and other effector genes [31,32].

In insects, infections with different microorganisms or parasites selectively activate various defence reactions often related to the activation of specific immune genes. The molecular basis for the recognition of different types of ‘non-self’ and the activation of elicitor-specific immune responses is attributed to the specificity of PRRs for PAMPs, such as lipopolysaccharides, peptidoglycan or various glucans [33,34]. Several proteins in insect hemolymph seem to act as PRRs since they perform surveillance by binding to molecular patterns common to groups of microorganisms [35]. Functions and molecular structure of several PPRs have been described including: peptidoglycan-receptor proteins, β-glucan receptors and immune-related sugar-receptors named immulectins [11,36–39].

In \textit{G. mellonella} naïve larvae, two LPS-binding proteins, namely LBP-1 (17.2 kD) and LBP-2 (26 kD), have been described by Dunphy and Halwani [40]; these receptors bind the surface of bacteria and apparently act as detoxifiers of endotoxins thus protecting hemocytes from damage. Both the LBPs are specific for the lipid A portion of LPS, and LBP-1 seems to play the role of an activator of \textit{Galleria} proPO system. In the same year, Wiesner and colleagues [41] isolated and described a similar protein with a molecular mass of 17 kD, called Apolipophorin-III (ApoLp-III), later identified as the LBP-1 described by Dunphy [40]. ApoLp-III, which has been shown to possess immune-stimulating properties, is an exchangeable apolipoprotein, abundant in lepidopteran insects. The immune-stimulating capacity of ApoLp-III is surprising, since this protein has been known to play an important role in lipid transport in flying insects. Injection of tissue-derived, as well as of recombinant Apol-p-III [42], from \textit{G. mellonella} into the hemocoele of untreated larvae is followed by a strong increase in antibacterial activity within the hemolymph. The induced antibacterial activity reaches the same intensity as that which can be maximally provoked by injecting bacteria. Thus, with this protein, a further endogenous mediator has been identified, which is involved in the regulation of insect immune responses.

As discussed in detail elsewhere [14,24], the partial characterization of \textit{G. mellonella} HIPs, estimated
molecular masses and evidence in the relevant scientific literature [43,44] strongly suggest that HIP17 could actually be the insect lipid-carrier Apolipophorin III, and HIP26 could be the LBP-2 described by Dunphy and Halwani [40]. The third HIP (35 kD) exhibits a molecular weight similar to the protease-like molecule scolexin. Although this protein has been well characterized at molecular level, its biological role is yet unknown, however, in M. sexta, scolexin is described as a serine protease [45] whose synthesis is stimulated by immune activation [46]. Commonly, serine proteases play critical roles in several invertebrate immune processes, mainly in the activation of other proteins by site-specific cleavage [47].

Parasite immunoevasion and immunodepression strategies often involve the parasite body-surface, which seems to play a key role in the interaction with the host environment [19]. Nematodes moult several times throughout their developmental cycle and each time they change body-surface with the formation of a new cuticle [48,49]; although a common model of nematode cuticle has been proposed [50], single species may have sharp differences in molecular organization and surface properties. This is particularly true for parasitic species (i.e. S. feltiae) that must interact with an unfavourable host environment. Furthermore, parasitic nematodes may easily adjust the composition and organization of the epicuticular external layer, depending upon the particular environment of each species [50]. Along with other surface and secreted molecules [20], the cuticle of parasitic nematodes seems to be involved in immunoevasion and suppression of host’s defences, as suggested also by Akhurst and Dunphy [51]; thus the nematodes’ body-surface is likely to play a crucial role in the parasite life-cycle.

S. feltiae body-surface is able to suppress either the host proPO system-mediated melanization or to escape hemocytes recognition by a disguise-like mechanism. These properties seem to result from the affinity of its epicuticular lipidic compounds for some hemolymph cells. C: hemocytes from lipase-treated cuticle and bacteria-injected larvae, no bacterial cells are observable. Treated cuticle were markedly encapsulated, arrowheads. In all the assays hosts were infected with E. cloacae and hemocytes were cultured 12 h after the injection. Bottom: the graph shows total bacterial count (bacteria 100 ml⁻¹) in the hemolymph from the samples showed in micrographs. B: hemolymph from bacteria-injected larvae; CB: hemolymph from cuticles and bacteria-injected larvae; CLB: hemolymph from lipase-treated cuticles. Bacterial number was determined by UFC counts; mean ± SE, n = 5.
proteins (HIPs) responsible for the activation of various immune processes and pathways.

Moreover, it is not clear why metazoan parasites interfere with antibacterial activity of their hosts and down-regulate antimicrobial gene expression to develop within the host, as discussed by Richman and Kafatos [52] for metazoan and protozoan parasite of mosquitoes and blackflies.

Our data strongly suggest that *Steinernema* body-surface is also responsible for the inhibition of antimicrobial peptides production; on approaching this study we suspected a possible interference of the parasite with the antibacterial inducible response of the host; this hypothesis is particularly relevant in the studied model, since these parasites, other than to escape host immune defences, seem to be able to down-regulate AMPs genes preventing the synthesis of antimicrobial factors potentially harmful for their symbiotic bacteria.

In insect hemolymph, the appearance of newly synthesized peptides with strong antimicrobial activity is detectable after bacterial challenge; usually these molecules have a molecular weight below 10 kD and are easily observable by conventional electrophoretic methods for small proteins. By means of these assays and the analysis of bacterial growth we ascertained the effects of the presence of *Steinernema* cuticle compounds on the host systemic antimicrobial response.

Since parasite cuticles, when injected into host hemocoel cavity depress immune responses such as proPO system and avoid cell encapsulation, we assume that the parasite could disguise itself as ‘self’, by coating its body surface with hemolymph proteins of the host. The effect of the capture of hemolymph factors seems to be at the extracellular level, upstream of the pathways (Toll and/or IMD) leading to antimicrobial responses.

The specific interaction of the parasite cuticle with some hemolymph components (HIP17, HIP26 and HIP35) was confirmed by the data obtained after cuticle treatments; lipidic moiety alteration (or removal) resulted in the loss of the capacity to bind these molecules [14], leading to the impairment of immunosuppressive functions. Lipase or methanol–chloroform treated cuticles were unable to prevent the antimicrobial response of *Galleria*. A further confirmation of the involvement of lipidic components was provided by the injection of extracted cuticle lipids, whose effects were comparable to those of whole cuticles.

Also, the role of HIPs in the activation of the antibacterial peptides pathway was demonstrated when HIPs, purified from parasite cuticles, were injected with bacteria in the hemocoel of parasite cuticle-challenged hosts. A complete recovery of the antimicrobial response was observed concurrent with the appearance of low molecular weight peptides in the hemolymph.

The specific mechanism for the removal of certain components such as the HIPs has yet to be determined. However, the fact that these proteins possess LPS-binding properties and thus have the ability to bind to the wall of Gram-negative bacteria [53], suggests their involvement as PRRs responsible for the modulation of several immune processes including antimicrobial response.

In addition, the observation of the cross-reaction of cuticle lipids with anti-LPS antibodies [14] is in agreement with the hypothesis of the interference of the parasite body-surface with the LPS-mediated activation of antimicrobial pathways.

A schematic model (proposed in Fig. 10) has been formulated from the data obtained; epicuticular lipids (PCLs) may act as PAMP-like molecules, but the interaction with the HIPs results in the removal of the latter from the insect hemolymph, thus preventing the formation of suitable molecular complexes possibly needed to trigger the inducible response of the host. The effect of host immunodepression induced by nematode compounds interference resulted in a higher host mortality percentage; these data are summarized in Table 1.

![Fig. 10. Proposed model of parasite interference in antimicrobial peptide synthesis.](image-url)

- Proposed model of parasite interference in antimicrobial peptide synthesis. A schematic model is suggested from the data obtained; the interaction between parasite cuticular PAMPs (PCL) and host PRRs (HIPs) followed by their removal from the hemolymph, could results in a negative control upstream the IMD/TOLL pathways that culminate in the loss of activation of NFκB family transcription factors. PCL: parasite cuticular lipids; P-PAMPs: parasite PAMPs.
The above suggestion is strengthened by recent evidence of the central role of molecules such as ApoLp-III (a putative member of HIPS) in immune activation; with regards to the role of host apolipoproteins, they are involved in cellular and humoral immune responses [42,54]. ApoLp-III binds to Gram-positive bacteria and to lipoteichoic acid (LTA) [55], and as in mammals, apoLp-III can also bind and detoxify LPS [56,40] and promote phagocytosis and encapsulation [43,53]; besides, ApoLp-III stimulates antibacterial activity in the hemolymph [42] and superoxide production of hemocytes [57]. Furthermore, the suggested binding of the serine protease scolexin (35 kD) to Low Density Lipoprotein particles (LDLp) [44], seems to affect the activation of hemocytes and/or fat body cells; in addition, scolexin synthesis is stimulated by immune activation [46].

From the data reported in this work and on the basis of evidence from the literature, we can conclude that the absence or reduction of these PRRs from host hemolymph due to their interaction with parasitic cuticular lipids represents an active interference process leading to the avoidance of the triggering of several immune processes, including antimicrobial response.

The last few years have witnessed a significant growth of information in the field of host–parasite interactions; in particular, factors other than proteins have emerged as major players in such interactions. Moreover, the interface between parasites and the innate immune system has recently attracted a great deal of attention from researchers in the foreseeable future as well; primarily for the purpose of understanding the mounting and modulation of host immune responses.

Finally, a better knowledge of nematocomplexes-insects relationships could provide an improvement of integrated pests management techniques, with the aim to drastically reduce the use of chemicals in agriculture.

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References


Table 1

Percent of dead larvae after Gram-negative infection assays

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<td>5 ± 1</td>
<td>6 ± 2</td>
<td>6 ± 1</td>
<td></td>
</tr>
<tr>
<td>CLB</td>
<td>3 ± 1</td>
<td>5 ± 1</td>
<td>5 ± 1</td>
<td></td>
</tr>
<tr>
<td>EMCB</td>
<td>18 ± 2</td>
<td>69 ± 3</td>
<td>91 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

T, control buffer-injected larvae; B, bacteria-injected larvae; CB, cuticle-injected larvae; CMCB, methanol–chloroform pretreated cuticles-injected larvae; CLB, lipase-treated cuticles-injected larvae; EMCB, injection of extracts from methanolchloroform treatments. Values are means ± SE, n = 5.


Schagger H, von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 1987;166:368–79.


