Growing Bacteria Shed Elicitors of *Drosophila* Humoral Immunity

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Key Words

Innate • Insect • Peptidoglycan

Abstract

It has been much debated how the *Drosophila* immune system can recognize bacterial peptidoglycan that is often hidden. We show that bacteria separated from *Drosophila* S2 cells by a semipermeable membrane can upregulate the Imd pathway. Supernatants from exponentially growing but not from stationary-phase bacterial cultures induce antimicrobial peptides. It is also made likely that the shed elicitors are of peptidoglycan nature.

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Introduction

The cellular insect immune defense involves phagocytosis of microorganisms by circulating plasmatocytes in the hemocoel. In addition to the prophenoloxidase cascade, the humoral response relies on antimicrobial peptides (AMPs) secreted from the fat body. AMPs are synthesized upon activation of either one or both of two immune pathways in the *Drosophila* immune system. The Imd and Toll pathways recognize peptidoglycan (PGN), a major constituent of bacterial cell walls, by peptidoglycan recognition proteins (PGRPs) [for review, see 1].

PGN consists of carbohydrate chains with alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues, which are bound to stem peptides that in turn are cross-linked, either directly or by interpeptide bridges [2]. With a DAP residue in the third position, PGN is a better inducer of the Imd pathway than PGN with a Lys residue in this position [3]. The binding of purified PGN to PGRPs is well established, but it is not clear how the PGN in live bacteria is recognized by the immune system since PGN is hidden by teichoic acids in Gram-positive bacteria and by an outer membrane in Gram-negative bacteria.

Bordetella pertussis and *Neisseria gonorrhoeae* are known to shed large amounts of the PGN monomer, tracheal cytotoxin (TCT) [4, 5]. However, most bacteria have an efficient recycling of PGN in which most turnover products are captured and reutilized [6]. To explain how PGN becomes visible for the immune system, it has been suggested that plasmatocytes would phagocytose bacteria and (1) either digest them and release small fragments of PGN, which in turn are presented to the immune receptors and/or (2) release cytokines that activate AMP synthesis in the fat body [7].

In the present study, we used *Drosophila* Schneider 2 (S2) cells as a model system to show that phagocytosis of bacteria is not necessary for a humoral immune response. Instead bacteria in exponential growth phase, but not in stationary phase, release PGN fragments that are the elicitors of the Imd pathway.

Materials and Methods

Bacterial Strains and Growth Conditions

Escherichia coli strain MG1655 [8], *Micrococcus luteus* M11 (strain A270; Pasteur Institute Collection) and *Enterobacter cloacae* strain B11 [9] were grown with aeration at 37°C in *Drosophila* Schneider cell medium (Lonza) with 10% fetal calf serum (FCS; HyClone) or defined minimal medium (CaCl₂ 0.1 mM, MgSO₄ 1 mM), 0.2% glucose, thiamine dichloride 0.01 µg/ml and M9). Bacteria grown overnight or to OD₅₉₀ = 0.5 were centrifuged for 10 min at 6,700 g and the supernatants were passed through a 0.45-µm sterile filter (Sarstedt).

Enzymatic Digestion of Bacterial Filtrate and Peptidoglycan

The filtrate from an exponentially growing *E. coli* culture in Schneider medium was incubated with *Mus musculus* PGRP-L (20 µg/ml) [10]. As a control, insoluble PGN from *E. coli* MG1655, prepared as described earlier [11], was suspended in 60 mM TRIS pH 8, 100 mM NaCl to a final concentration of 0.5 mg/ml and incubated with *M. musculus* PGRP-L (20 µg/ml). The filtrate from *M. luteus* was first incubated with hen egg white lysozyme (10 µg/ml; Sigma) for 1.5 h at 37°C followed by incubation with PGRP-L (20 µg/ml) overnight at room temperature. Before addition to the cells, FCS was added to the medium to a final concentration of 10% and the medium was made isotonic by addition of NaCl.

Immune Stimulation of S2 Cells with Bacterial Growth Media

S2 cells were grown in 35-mm culture dishes in Schneider's *Drosophila* medium (Lonza) supplemented with 10% FCS (HyClone) and 50 mg/ml of penicillin G and 50 mg/ml of streptomycin sulfate. Cells were seeded at 1.5×10^6 cells/ml and grown overnight at 25°C. The cells were challenged by exchanging the cell medium for 3 ml of filtrates from *E. coli*, *M. luteus* and *E. cloacae* grown overnight or to OD₅₉₀ = 0.5. Cell viability was estimated using 0.2% Trypan blue and a hemocytometer. Undigested and *M. musculus* PGRP-L digested filtrates from an exponentially growing *E. coli* were added to the S2 cells under the same conditions.

Immune Stimulation in S2 Cells Co-Cultivated with Bacteria

S2 cells were grown as described above but without penicillin G and streptomycin sulphate in dishes with polycarbonate membrane inserts, pore size 0.4 µm (Corning). Cells were seeded to a density of 4.5×10^6 per well or insert and grown at 25°C. *E. coli* were grown to OD₅₉₀ = 0.5 and added to a final concentration of 4.5×10^6 bacteria/well or insert, giving a multiplicity of infection of 0.5 as the cell number had doubled during the overnight incubation.

Exponentially grown bacteria were heat killed at 65°C for 30 min, washed and resuspended in *Drosophila* Schneider medium. Viable counting showed that the viability had decreased at least three orders of magnitude.

RNA Preparation and Quantitative RT-PCR

Total RNA was isolated following 3 h of immune stimulation using Trizol (Invitrogen) according to the manufacturer's protocol. DNA was removed using the TURBO DNA-free™ kit (Ambion) and cDNA was synthesized by RT-PCR using the qScript cDNA Synthesis Kit (Quanta).

Quantitative RT-PCR was performed using a LightCycler®480 II Real-Time PCR system (Roche) or a PrismP 7000 Real-Time PCR System (Applied Biosystems). Primer and probe sequences: *AttacinA*, 5'-CCGCCGGAACACTCAAA-3' (forward), 5'-GG-GCC TCCTGCTGGAAA-3' (reverse) and 6-FAM-CAACAAT-GCTGGTCATGGT; *ribosomal protein 49*, 5'-CACCAGTCGG-ATCGATATGCT-3' (forward), 5'-ACGCACTCTGTTGTCGA TACC-3' (reverse) and VIC-CATTTGTGCGACAGCTT or JOE-CATTTGTGCGACAGCTT.

Purification of Immune Stimulatory Compounds from *E. coli* Minimal Medium

The supernatant from *E. coli* grown to exponential phase was applied to a Strata C18 E solid-phase extraction column (Phenomenex). Elution was with 20% acetonitrile containing 0.01% trifluoroacetic acid (TFA). The samples were evaporated to dryness in a Speed-vac concentrator and resuspended in milli-Q water. The resuspended material was further purified by reversed-phase HPLC (Dionex) using a Strata Gemini 5 µ C-18 column, 150 × 3.00 mm (Phenomenex).

Mass Spectrometry

The sample was resolved in 50% ACN/0.2% HCOOH and introduced from a metal-coated borosilicate glass capillary needle (Proxeon Biosystems A/S) on a QTOF Ultima API mass spectrometer (Waters Corp.) equipped with a standard Z-spray API source. The mass spectrometer was operated in positive mode at a resolution of 10,000, full with at half maximum height definition. The collision gas was Argon with a pressure of 5.5×10^{-5} mbar in the analysis-penning read back. The capillary voltage was 1.5 kV. Data were acquired between 100 and 1,000 m/z with a scan time of 1 s for about 5 min. The spectra were combined in Masslynx 4.1 software (Waters Corp.).

Statistical Analysis

The relative expression values were logarithmically transformed to make the data more normally distributed and the means were tested for significant differences using ANOVA and Bonferroni's all-pairs comparisons as post hoc test ($p < 0.01$).

Results

Bacterial Contact Is Not Needed to Upregulate the *Imd* Pathway in S2 Cells

S2 cells and *E. coli* were grown simultaneously, separated by a semipermeable membrane in order to avoid physical interaction and to prevent phagocytosis. *E. coli* separated from S2 cells are able to induce *attacinA*, a read-out of the *Imd* pathway in this system (fig. 1). Thus, phagocytosis of bacteria is not a requirement for an immune response in S2 cells. The immune response was not enhanced by the presence of S2 cells together with the bacteria in the upper chamber (bar 4). Typically, 20% of such S2 cells have internalized bacteria (not shown), suggesting that phagocytosing cells in the insert do not re-

lease signals to increase the immune response of the cells in the well. The heat-killed bacteria (bar 5) do not release any elicitors and consequently have to be in close contact with the cells to elicit the immune response [12]. The stronger induction of *attacinA* in S2 cells in direct contact with bacteria could be due to the facilitated diffusion of elicitors in the absence of a membrane and to the contact itself.

The Supernatant from E. coli in Exponential Growth Phase Can Induce the Imd Pathway

The previous experiment indicates that growth media from commonly used bacteria should induce AMPs. The supernatants from both overnight and exponentially grown cultures of *E. coli* were centrifuged and filtered through a 0.45- μ m filter and added to S2 cells (fig. 2a). The filtrate from a bacterial culture grown overnight shows a very low induction of *attacinA*. However, supernatant from *E. coli* in exponential growth phase showed to be a strong elicitor of the Imd pathway. Thus, exponentially growing *E. coli*, but not *E. coli* in stationary phase, release elicitors that are able to trigger the immune response in S2 cells. Even if the viability of the S2 cells after being in contact with the stationary-phase medium is typically 90%, we wanted to test if the cells still are immunocompetent. Cells were thus incubated for 1 h with medium from bacteria in stationary phase and then for 3 h with medium from exponentially growing bacteria (bar 4). This treatment did not result in a lower response, indicating that the stationary-phase medium has no detrimental effect on immunocompetence.

To rule out the possibility that the release of immune-activating substances is media dependent, *E. coli* was cultured in a defined minimal medium. This filtrate elicits a similar pattern of *attacinA* expression, as did the conditioned *Drosophila* Schneider medium (data not shown).

Both Gram-Positive and Gram-Negative Bacteria in Exponential Growth Phase Release Fragments of PGN Nature That Can Induce the Imd Pathway

To test if the elicitor shedding is general among bacteria, growth media from both Gram-negative and Gram-positive bacteria were tested. The Gram-negative *E. cloacae*, having DAP-type PGN, and the Gram-positive *M. luteus*, having Lys-type PGN, both show similar elicitor patterns, that is, a release of immune-stimulatory compounds during growth but not when they have reached stationary phase (fig. 2b). The viability of the S2 cells after 3 h of incubation was around 90%.

Fig. 1. Induction of *Drosophila* S2 cells with live or heat-killed *E. coli*. S2 cells were grown in plates with inserts having semipermeable bottoms. Cells were unchallenged (bar 1), challenged with live growing *E. coli* bacteria (bars 2–4) or with heat-killed *E. coli* (bar 5) to give a multiplicity of infection of 2 at the end of the 3-hour incubation. The location of cells (green/large circles) and bacteria (red/small circles) in the different wells and inserts are shown in schematic drawings below the diagram. After incubation total RNA was isolated from the cells in the wells. The mean values (+SEM) of relative *attacinA* levels from 3–6 real-time RT-PCR assays run in triplicates are shown. Values are relative to that of the sample with bacteria separated from the S2 cells (bar 3). * $p < 0.01$ versus both those of bar 1 and 5 but not from each other.

As purified PGN is the only well-documented bacterial component being an inducer, we tested if the released compounds were of PGN nature. The *M. musculus* PGRP-L amidase, which cleaves the bond between the carbohydrate chain and the stem peptide, including this bond in the terminal anhydromuramoyl peptide, can abolish the immune-stimulatory effect of PGN [10, 13]. The filtrate from exponentially growing *E. coli* treated with *M. musculus* PGRP-L shows decreased levels of *attacinA* induction, as did the treated *E. coli* PGN (fig. 3a). Thus, the released elicitor from *E. coli* is most likely of PGN nature considering the high specificity for PGN of this amidase. Lysozyme and *M. musculus* PGRP-L can be used to degrade Gram-positive PGN [10]. The low *attacinA* induction observed after treating the supernatant with these enzymes indicates that also the *Micrococcus* elicitor is of PGN nature (fig. 3a).

To further characterize the elicitor compound, the supernatant from *E. coli* grown to exponential phase in minimal medium was concentrated and separated by

Fig. 2. a Filtrate from *E. coli* grown to $OD_{590} = 0.5$ can induce *attacinA* in *Drosophila* S2 cells. S2 cells were grown in 6-well plates and stimulated by replacing the *Drosophila* medium (bar 1) with either the filtrate from exponentially growing *E. coli* (bar 2) or from *E. coli* in stationary phase (bar 3). The S2 medium was also replaced with filtrate from *E. coli* in stationary phase for 1 h and then with filtrate from exponentially growing *E. coli* for 3 h (bar 4). As a positive control, PGN (5 μ g/ml) was used (bar 5). **b** Filtrates from *E. cloacae* and *M. luteus* can also induce *attacinA* expression in *Drosophila* S2 cells. S2 cells were challenged as in

a with filtrate from *E. cloacae* grown to exponential phase (bar 3) or stationary phase (bar 4) or *M. luteus* grown to exponential phase (bar 5) or stationary phase (bar 6). As control, filtrate from *E. coli* in exponential phase (bar 2) was used. The mean values ($\pm$ SEM) of relative *attacinA* levels from 3–5 real-time RT-PCR assays run in triplicates are shown. Values are relative to that of the sample with medium from exponentially growing *E. coli* (bar 2). * $p < 0.01$ versus those of bars 1 and 3 (**a**) and versus those of bars 1, 4 and 6 (**b**), but not versus each other.

HPLC as described in Materials and Methods (fig. 3b). The peaks marked 1 and 2 resulted in a 3.1- and 5.4-fold induction of *attacinA*, respectively, compared to that of the corresponding fractions of the control. The two fractions were subjected to mass spectrometry. A peak with m/z 922.35, corresponding to the molecular mass of 922 Da of TCT ($M+H$)⁺, in fraction 1 gave further support to the conclusion that the elicitor is of PGN nature (fig. 3c). This peak was too minor to allow further structure determination by MS/MS.

Discussion

PGN has been shown to be the major bacterial elicitor of both the Toll and Imd pathways. The interactions between PGN and its PGRP receptors have been determined both regarding receptor structure and elicitor specificity using purified components. The actual presentation of the PGN by the bacterial invader is less un-

derstood. It has been reported that PGN fragments signal bacterial contamination over long distances both in *Drosophila* [14] and mice [15]. Our results now show that exponentially growing bacteria shed PGN fragments in quantities sufficient to induce AMP genes. These results are obtained in vitro using S2 cells as a proxy for fat body cells. This might be a justified model for AMP induction as both cell types express PGRP-LC, which is sufficient to start an Imd-mediated immune response. PGRP-LC has affinity for both DAP- and Lys-type PGN [16] and S2 cells do not express PGRP-LE, a receptor of the Imd pathway with preference for DAP-type PGN [17], properties that might result in S2 cells being less DAP specific than fat body cells.

The PGN fragments released by lytic enzymes during growth are captured and reutilized in most bacteria [6]. Even so, the levels of elicitor released into the supernatant of exponentially growing *E. coli* cultures are sufficient to induce an immune response in *Drosophila* S2 cells. A possible explanation of the lack of elicitor activity in station-

Fig. 3. The elicitors are of PGN nature. **a** The eliciting effect can be reduced by PGN-degrading enzymes. *Drosophila* S2 cells were stimulated by replacing the *Drosophila* cell medium (bar 1) with either the filtrate from exponentially growing *E. coli* (bar 4) or filtrate from exponentially growing *E. coli* pre-incubated with *M. musculus* PGRP-L (bar 5). S2 cells were also challenged with filtrate from exponentially growing *M. luteus* (bar 6) or filtrate from exponentially growing *M. luteus* pre-incubated with PGN-degrading enzymes as described in Materials and Methods (bar 7). As controls, *E. coli* peptidoglycan (5 μ g/ml) was used untreated (bar 2) or pre-incubated with *M. musculus* PGRP-L (20 μ g/ml) for 15 h and thereafter added to the cells (bar 3). The cells were incubated for 3 h and total RNA was isolated. The mean values (+SEM) of relative *attacinA* levels from 3–6 real-time RT-PCR assays run in triplicates are shown. Values are relative to those of the sample

with untreated filtrate from *E. coli* in exponential phase (bar 4). **b** HPLC separation of filtrate from *E. coli* grown to exponential phase in a defined minimal medium. The growth medium was enriched as described in Materials and Methods and further analyzed using HPLC. Elution was with a gradient of 0–60% acetonitrile containing 0.01% TFA, at a flow rate of 0.5 ml/min and with monitoring at 206 nm. Trace a is from bacteria-conditioned medium and trace b is from control medium treated similarly. Fractions were evaporated to dryness in a Speed-vac concentrator and re-suspended in *Drosophila* Schneider medium and the degree of immune stimulation was tested by challenging *Drosophila* S2 cells as described in Materials and Methods. Only the fractions marked 1 and 2 resulted in upregulation of *attacinA*. The arrow indicates the elution position of pure TCT. **c** The full scan mass spectrum of fraction 1 in **b**.

ary-phase medium could be an even more efficient recycling of PGN during starvation conditions. Gram-positive bacteria, in contrast to Gram-negative, have a higher loss of PGN during growth [18]. Our induction results are in consonance with this fact. The induction is consequently dependent on both PGN type and the amount of the released fragment. As only dividing bacteria induce a response, the strength of a bacterial species as an AMP elicitor is dependent on how much the immediate early arms of innate immunity (phagocytosis, nodule formation and phenoloxidase) allow the bacterium to divide. This illustrates yet another parameter influencing the selectivity of Toll- and Imd-mediated AMP induction.

Our results might offer an explanation to the observations by Lindmark et al. [19]. They found that very high numbers of live *E. coli* abrogated the immune response elicited by LPS. The rather low amount of PGN impurity in LPS preparations that constitute the actual elicitor in that experiment might have been absorbed and reutilized by the high numbers of added live stationary-phase bacteria, thus abrogating the immune activation caused by the added LPS (PGN).

Hemocytes remove most of the bacterial cells within the first hour of infection before the AMPs do the ‘mopping up’ of the remaining bacteria [20]. Most studies using *Drosophila* mutants have refuted phagocytosis as a

prerequisite for systemic AMP induction, for example, an eater mutant, which lacks a phagocytosis receptor and hemocyte-less flies, both show a normal humoral immune response [21–23]. Thus, the removal of most bacteria by phagocytosis and the lack of contribution from neither these removed bacteria nor the phagocytes to the AMP induction in the fat body underline the importance of an AMP induction being mediated by PGN fragments shed by growing free bacteria, of the type that we demonstrate.

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Disclosure Statement

We report no conflict of interest.

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