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Enteric Bacteria Counteract Lipopolysaccharide Induction of Antimicrobial Peptide Genes

Hans Lindmark,* Karin C. Johansson,* Svenja Stöven,† Dan Hultmark,† Ylva Engström,‡ and Kenneth Söderhäll**

The humoral immunity of *Drosophila* involves the production of antimicrobial peptides, which are induced by evolutionary conserved microbial molecules, like LPS. By using *Drosophila* mnb-2 cells, we found that live bacteria, including *E. coli*, *Salmonella typhimurium*, *Erwinia carotovora*, and *Pseudomonas aeruginosa*, prevented LPS from inducing antimicrobial peptide genes, while *Micrococcus luteus* and *Streptococcus equi* did not. The inhibitory effect was seen at bacterial levels from 20 per mnb-2 cell, while antimicrobial peptides were induced at lower bacterial concentrations (≤2 bacteria per cell) also in the absence of added LPS. Gel shift experiment suggests that the inhibitory effect is upstream or at the level of the activation of the transcription factor Relish, a member of the NF-κB/Rel family. The bacteria have to be in physical contact with the cells, but not phagocytosed, to prevent LPS induction. Interestingly, the inhibiting mechanism is, at least for *E. coli*, independent of the type III secretion system, indicating that the inhibitory mechanism is unrelated to the one earlier described for YopJ from *Yersinia*. The Journal of Immunology, 2001, 167: 6920–6923.

Production of antimicrobial peptides is one part of the innate immunity in invertebrates as well as in mammals and plants. Signaling cascades leading to expression of antimicrobial peptides are triggered by extracellular host molecules recognizing microbial molecules, like LPS, peptidoglycan, and β-1,3 glucan, called pathogen-associated molecular patterns (1). The fruitfly *Drosophila melanogaster* produces at least seven distinct antimicrobial peptides (cecropin, attacin, diptericin, insect defensin, drosocin, drosomycin, and metchnikowin). The expression of the peptide genes is in accordance with their antimicrobial effect; i.e., antifungal peptides are more induced by fungi whereas antibacterial peptides are more induced by bacteria (2–4). The promoters of all seven genes contain κB sites, which are binding sites for transcription factors of the NF-κB/Rel family. These factors are dimers of Rel proteins and in mammals they play an important role in the onset of an inflammatory response, because they enhance production of proteins like cytokines, acute phase response proteins, and adhesion molecules (5). Dorsal, Dif, and Relish are the known members of the Rel family in *Drosophila*. Relish seems to be the Rel protein that contributes most to the expression of antibacterial peptides (6) while both Relish and Dif play a pivotal role during induction of antifungal peptides (4, 6–8). The recent findings that bacteria are able to counteract induction of various host defenses (9–13) and that antibacterial peptides were induced at lower bacterial concentrations (2–4). The promoters of all seven genes contain κB sites, which are binding sites for transcription factors of the NF-κB/Rel family. These factors are dimers of Rel proteins and in mammals they play an important role in the onset of an inflammatory response, because they enhance production of proteins like cytokines, acute phase response proteins, and adhesion molecules (5). Dorsal, Dif, and Relish are the known members of the Rel family in *Drosophila*. Relish seems to be the Rel protein that contributes most to the expression of antibacterial peptides (6) while both Relish and Dif play a pivotal role during induction of antifungal peptides (4, 6–8). The recent findings that bacteria are able to counteract induction of various host defenses (9–13) and that antibacterial peptides were induced at lower bacterial concentrations (2–4.

**Materials and Methods**

**Cell line and bacterial strains**

The *Drosophila* tumorous blood cell line mnb-2 was cultivated and infected essentially as previously described (15), although antibiotics were not used. The *Salmonella typhimurium*, *Escherichia coli*, *Streptococcus equi*, and *Pseudomonas aeruginosa* are clinical animal isolates obtained from the National Veterinary Institute (Uppsala, Sweden); *Erwinia carotovora* SCC3193 was a gift from M. Pirhonen (Swedish University of Agricultural Sciences, Uppsala, Sweden) and the *Micrococcus luteus* strain was provided by H. Boman (Karolinska Institute, Stockholm, Sweden). *E. coli* CVD 452, lacking an essential gene (sepB) for the type III secretion system, together with its parental strain E2348/69 were obtained from M. Donnenberg and J. Kaper (University of Maryland School of Medicine, Baltimore, MD). Bacteria were cultured in Luria-Bertani or tryptic soy broth at 37°C, except *E. carotovora*, which was cultured at 28°C.

**Infection experiments**

Bacterial overnight cultures were diluted 1/50 and cultivated to an OD_{600} of 0.5. One sample from each bacterial species was inactivated by incubating 10 ml of the culture in a 55°C (*S. equi*), 65°C (*E. coli*, *S. typhimurium*, and *E. carotovora*), or 70°C (*M. luteus*) waterbath for 15 min. After centrifugation, the bacterial cells were resuspended in Schneider medium and used for infecting the mnb-2 cells at a multiplicity of infection (MOI) of 20. In appropriate cases LPS (10 μg/ml, O127:B8, Sigma-Aldrich, St. Louis, MO) was added simultaneously with the bacteria to the mnb-2 cells. After 6 h of incubation, the mnb-2 cells were harvested and total RNA was extracted, using TRZol reagent (Life Technologies, Rockville, MD). Northern blots were performed in accordance with standard protocols. The probes used were as follows: *Diptericin* cDNA (16), *Cecropin A1* cDNA (17), *Drosomyacin* cDNA (18), a PCR-generated probe (724 bp) for *Drosocin* based on published sequence (19), a PCR-generated probe (1396 bp) for GAPDH1 based on published sequence (20), a PCR-generated probe for ferritin (f-3TR) (21), and a PCR-generated probe for *rp49* (22), the latter probe was used as a loading control.

**EMSA**

The mnb-2 cells were infected as described above and nuclear extracts were prepared as previously described (23). The protein concentration in the nuclear extracts was determined by the Bradford assay. Binding reactions were conducted by mixing 0.5 ng of a 32P-labeled probe (5'-GTGA CATTGGGATTCCCTTTTGCA) containing a κB site present in the *Diptericin* promoter (24), 10 μg of nuclear extract, 60 μg BSA, and 1 μg

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of poly(dI·dC) in 20 µl of a buffer containing 20 mM HEPES, 100 mM NaCl, 1 mM EDTA, and 8.5% (v/v) glycerol. A Relish Ab (25) was in appropriate cases added to the nuclear extracts immediately before addition of the xB probe. After 15 min, incubation at room temperature EMSA was conducted as described elsewhere (26).

**Experiments using transwells and cytochalasin D**

Infection of mbn-2 cells was essentially conducted as described above. Transwells (Costar, Cambridge, MA) were used; these have a permeable (0.4 µm) membrane situated close to the bottom of the well preventing added bacteria from coming in direct contact with the mbn-2 cells seeded below the membrane (see Fig. 5B). Cytochalasin D (5 µM, Sigma-Alt- drich), dissolved in DMSO, was added 30 min before bacterial infection to prevent phagocytosis. The final concentration of DMSO in the wells was 2.5 µM. That cytochalasin D inhibited phagocytosis was controlled microscopically using FITC-labeled heat-inactivated *E. coli*.

**Silencing and reinduction of Diptericin**

mbn-2 cells were infected with live *E. coli* (MOI 20) and 10 µg/ml LPS and after 2 h of incubation, the cells were washed with 1× PBS-D and resuspended in growth medium containing 10 µg/ml LPS, 200 µg/ml gentamicin, and 200 µg/ml streptomycin. Northern blot was performed after incubation for the times indicated. Gentamicin and streptomycin were controlled not to induce *Diptericin* by themselves.

**Results**

**Bacterial species affect the expression of antimicrobial peptide genes differently**

Six bacterial species were tested for their ability to counteract LPS induction of four antimicrobial peptide genes, using the *Drosophila* blood cell-line mbn-2 (Fig. 1). Beside *S. equi*, all heat-inactivated bacteria strongly induced the three antibacterial peptide genes *Diptericin*, *Drosocin*, and *Cecropin A1* while *Dromycin*, an antifungal peptide gene, was less induced. That neither live nor dead *S. equi* induced any antimicrobial peptide gene is noteworthy because this bacterium normally gives rise to a strong inflammatory response in mammals (27). Live *E. coli*, *S. typhimurium*, *E. carotovora*, and *P. aeruginosa* did not induce any of the four antimicrobial peptide genes tested. Interestingly, although LPS is a strong elicitor of antimicrobial peptide genes, no induction of the antimicrobial peptide genes occurred when LPS was added together with any of the four enteric bacteria to the mbn-2 cells (Fig. 1). This suggests that these bacteria secrete a component that blocks the signal transduction pathway, initiated by the recognition of LPS and culminating in enhanced expression of antimicrobial peptide genes. It seems that the tested enteric bacteria have less ability to inhibit induction of *Cecropin* compared with the other antimicrobial peptides and that this phenomenon is more pronounced for *Pseudomonas* (Fig. 1). To test whether any serum component affected the obtained results we exchanged the serum-containing growth medium to Ultimate Insect Serum-Free medium (Invitrogen, San Diego, CA). The obtained results were identical for the two growth media.

**Live *E. coli* inhibits activation of Relish**

To investigate the mechanism that counteracts LPS induction of antimicrobial peptide genes we focused on *E. coli* and the antibacterial peptide gene *Diptericin*. EMSA experiments were performed to elucidate whether the *E. coli* component counteracts activation of Rel proteins. LPS and heat-inactivated *E. coli* strongly activated Rel proteins with affinity to a xB site present in the *Diptericin* promoter whereas live *E. coli* or LPS plus live *E. coli* did not activate Rel proteins (Fig. 2). A Relish-specific Ab shifted the whole single band displayed, indicating that Relish constitutes at least one part of the dimer that forms a complex with the xB-probe (Fig. 2).

**The effect of different incubation times and bacterial concentrations**

Time studies showed that live *E. coli* immediately starts to suppress LPS induction of *Diptericin* and that the induction is suppressed during the whole incubation (Fig. 3). How different bacterial concentrations affected the inhibitory effect on LPS induction was also tested and as seen in Fig. 4 the inhibitory effect disappears when the bacterial inoculum is lowered to 10^3 CFU, corresponding to a MOI of 2 bacteria per cell. When the experiments in Fig. 4 were repeated without adding free LPS to the wells we found that

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**FIGURE 1.** Northern blots with total RNA extracted from bacteria-infected mbn-2 cells. Two identical blots were produced that were successively hybridized with radioactively labeled probes covering *Diptericin*, *Drosomycin*, *Drosocin*, *Cecropin A1*, and *rp49*. Bacteria (10^6 CFU) are *E. coli* (Ec), *S. equi* (Se), *E. carotovora* (Ec), *M. luteus* (Ml), *P. aeruginosa* (Pa), *S. typhimurium* (St), *Cecropin A1* (Cec A1), and *rp49*. Lane C, uninfected; lane L, LPS from *E. coli*; lane 1, live; lane 2, heat inactivated; lane 3, live + LPS.

**FIGURE 2.** *E. coli* counteracts LPS activation of Relish. EMSA using nuclear extracts from *Drosophila* mbn-2 cells and a probe covering a xB motif present in the *Diptericin* promoter. Lane 1, uninfected; lane 2, LPS; lane 3, LPS + Relish Ab; lane 4, live *E. coli*; lane 5, heat-inactivated *E. coli*; lane 6, heat-inactivated *E. coli* + Relish Ab; lane 7, live *E. coli* + LPS (7).

**FIGURE 3.** LPS induction of *Diptericin* is suppressed by *E. coli* directly after infection and the amount of *Diptericin* RNA remains low during the whole period of incubation. mbn-2 cells were incubated with LPS (●) or LPS + live *E. coli* (■) and the amount of *Diptericin* transcript was quantified at different times by Northern blot. Points represent means of duplicates, and SD are indicated.
10^7, 10^6, and 10^5 CFU of live E. coli strongly induced Diptericin while 10^6 CFU did not (data not shown). Thus, the innate immune system recognizes live E. coli, but at a MOI of 20 and higher the bacterial inhibitory effect suppress the induction of Diptericin. The inhibitory effect is independent of the type III secretion system

Blocking phagocytosis by addition of cytochalasin D and inhibiting direct physical contact between bacteria and cells by a membrane showed that the bacterium has to be in physical contact with the host cell but not necessarily phagocytosed to prevent LPS induction of Diptericin (Fig. 5, A and B). These results suggested that the E. coli inhibitory factor is secreted by a type III secretion system. Many pathogenic Gram-negative bacteria, including the four species in this study, use a type III secretion system to translocate proteins directly into the cytosol of host cells (28). Once inside the host cell, the bacterial proteins start to manipulate the host cell in a way that is beneficial for the bacterium. However, an E. coli mutant, CVD 452, lacking an essential gene for the type III secretion system was found to inhibit LPS induction of Diptericin as much as its parental strain 2348/69 (Fig. 5C). In addition the transwell experiment shows that the bacterial inhibitory effect is not due to changes in the medium caused by active microbial metabolism or oxygen depletion because infection with 10^9 CFU of live E. coli did not show any significant reduction in the inducibility of Diptericin (Fig. 5B).

The inhibitory effect on LPS induction is specific and reversible Northern blot experiments showed that transcription from nonimmune genes was unaffected by live E. coli (Fig. 6). This shows that E. coli does not generally increase the mRNA turnover but specifically counteracts LPS induction. Furthermore, we investigated whether the E. coli inhibitory effect is reversible. mbn-2 cells were infected with LPS and live E. coli and after 2 h of incubation the wells were washed once to reduce the number of bacteria. The cells were further incubated with fresh medium containing LPS and antibiotics. Northern blot showed that E. coli-infected cells had induced Diptericin 2 h postwashing (Fig. 7). Thus, the inhibitory effect is reversible and cells expressing antimicrobial peptide genes are not killed by E. coli.

Discussion

To avoid recognition and develop resistance against the host’s armed forces are two strategies microbes use to cope with the host immune system. Examples of a third strategy, to actively interfere with signaling pathways leading to activation of the immune system has recently been demonstrated for several bacteria-host interactions (9–13). For example, E. coli inhibits phagocytosis by injecting, into the cytosol of the host, proteins that dephosphorylate signaling proteins necessary for phagocytosis. Yet another example is the enteric bacterium Yersinia which counteracts induction of proinflammatory genes by secreting a protein, Yop J, through the type III secretion system. Inside the host cell, Yop J binds to IKKβ, and thereby is phosphorylation of I-κB, a step in

FIGURE 4. A MOI of 20 is needed for E. coli to suppress LPS induction of Diptericin. mbn-2 cells were infected with LPS + various amounts of E. coli. The amount of Diptericin was quantified by Northern blot. The bars represent means of duplicates, and SD are indicated.

FIGURE 5. Northern blots showing that E. coli has to be in physical contact but not phagocytosed to inhibit LPS induction of Diptericin. A. The effect of cytochalasin D on Diptericin expression. Lane 1, untreated; lane 2, LPS; lane 3, live E. coli; lane 4, dead E. coli; lane 5, LPS + live E. coli. Lanes marked with + indicate that the cells were pretreated with cytochalasin D. B. Transwells with mbn-2 cells seeded in the lower compartment were infected as indicated. The inoculum of live E. coli was 10^9 CFU. C, A type III secretion mutant inhibits LPS induction of Diptericin. mbn-2 cells infected with LPS and various amounts of E. coli. Lane 1, 10^9 CFU; lane 2, 10^7 CFU; lane 3, 10^5 CFU, WT (wild type), and CVD (mutant lacking type III secretion system)

FIGURE 6. The expression of nonimmune genes is unaffected by live E. coli. Northern hybridization of total RNA. Lane 1, untreated; lane 2, LPS; lane 3, live E. coli; lane 4, dead E. coli; lane 5, LPS + live E. coli.

FIGURE 7. The E. coli inhibitory effect on LPS induction of Diptericin is reversible. mbn-2 cells were infected with LPS + live E. coli and after 2 h of incubation the growth medium was replaced by new medium containing LPS and antibiotics. After further incubation the cells were harvested and the amount of Diptericin RNA was measured by Northern blot. Lane 1, 2 h of infection; lane 2, 6 h of infection; lane 3, 2 h of infection and 2 h incubation after washing; lane 4, 2 h of infection and 4 h incubation after washing; lane 5, 2 h of infection and 6 h incubation after washing; lane 6, 2 h of infection and 8 h incubation after washing.
activation of NF-κB blocked (12). That the bacterial mechanism suppressing LPS induction described here is similar to the one in Yersinia is unlikely because no type III secretion system is required for E. coli. Recently, it was shown that nonpathogenic Salmonella (S. Pullorum) is capable of attenuating the IL-8 secretion characteristically elicited by TNF-α and pathogenic S. typhimurium (13). The inhibitory effect was also in this case achieved by inhibiting activation of NF-κB. However, inhibition of NF-κB activation is in this case not reached by blocking phosphorylation of IκB but by inhibiting the subsequent step, namely polyubiquitination of IκBα (9). Whether the anti-inflammatory mechanism of S. Pullorum is related to the one here described for enteric bacteria is today an open question.

Like mammals, insects possess a circulatory system and a complex innate immune response. Recent data have shown that pathogen recognition, signaling pathways, and effector mechanisms of innate immunity are conserved between Drosophila and mammals (29). Our finding that enteric bacteria normally present in mammals suppress the immune system in an insect show that also the interplay between enteric bacteria and the innate immune system is evolutionarily conserved. Taken together, this suggests that Drosophila is a useful model for studying how microbes interact with the innate immune system of higher organisms.

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