Fungal Peptide Destruxin A Plays a Specific Role in Suppressing the Innate Immune Response in Drosophila melanogaster

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Destruxins are a class of insecticidal, anti-viral, and phytoxic cyclic depsipeptides that are also studied for their toxicity to cancer cells. They are produced by various fungi, and a direct relationship has been established between Destruxin production and the virulence of the entomopathogen Metarhizium anisopliae. Aside from opening calcium channels, their in vivo mode of action during pathogenesis remains largely uncharacterized. To better understand the effects of a Destruxin, we looked at changes in gene expression following injection of Destruxin A into the fruit fly Drosophila melanogaster. Microarray results revealed reduced expression of various antimicrobial peptides that play a major role in the humoral immune response of the fly. Flies co-injected with a non-lethal dose of Destruxin A and the normally innocuous Gram-negative bacteria Escherichia coli, showed increased mortality and an accompanying increase in bacterial titers. Mortality due to sepsis was rescued through ectopic activation of components in the IMD pathway, one of two signal transduction pathways that are responsible for antimicrobial peptide induction. These results demonstrate a novel role for Destruxin A in specific suppression of the humoral immune response in insects.

Insects are the most diverse and prolific land animals, and a variety of pathogens have specialized to infect them. Unlike bacteria or viruses that usually need to be ingested, certain fungal species can directly breach the insect cuticle to cause disease. Fungi are the most commonly observed insect pathogens in nature, causing the largest percentage of deaths because of infection. As a result, methods of controlling insect populations using live fungal insecticides have attracted medical and agricultural interest (1, 2). The ascomycete Metarhizium anisopliae is already in commercial use to control termites, grasshoppers, and thrips (2–4). In some fungi, success in infecting a wide variety of insects can, at least in part, be attributed to secretion of virulence factors during pathogenesis. Destruxins were initially identified as toxic compounds secreted by Metarhizium and were later characterized as important virulence factors accelerating the deaths of infected insects (5–8).

Chemically, Destruxins are cyclic hexadepsipeptides composed of an α-hydroxy acid and five amino acid residues. Five natural analogues (labeled A–E) have been isolated (5, 9, 10). These forms differ in the R-group of the hydroxyl acid residue and appear to have overlapping but different biological effects. Primarily, however, injection, ingestion, or topical application of a Destruxin on insects causes tetanic paralysis (11). Destruxin-induced membrane depolarization due to the opening of Ca2+ channels has been implicated as a cause of paralysis and death (11). Destruxin also causes signaling changes through the phosphorylative activation of certain proteins in lepidopteran and human cell lines. In addition, Destruxins cause morphological and cytoskeletal changes in insect plasmatocytes in vitro, and this adversely affects insect cellular immune responses, such as encapsulation and phagocytosis (12–14). These could be indirect results of a calcium influx (15).

Destruxins also show biological activities against non-insects. They are particularly toxic to mammalian leukemia cells and spleen lymphocytes and have demonstrated anti-proliferative activity on mouse neoplasms in vitro (16). Destruxins A, B, and E have also been shown to have antiviral properties in insect and human cell lines (17–19). For example, Destruxin B has demonstrated a suppressive effect on hepatitis B surface antigen expression (20, 21).

Aside from their ability to open calcium channels, the mechanisms by which Destruxins achieve their varied biological activities have not been studied in vivo. Therefore, we used Drosophila melanogaster as an insect model to characterize the range of functions affected by Destruxins. Among insects, Drosophila has the best characterized immune response, and because of similarities in key signaling pathways, has been an invaluable model for understanding innate immunity in humans. Flies have three active innate immune mechanisms for dealing with an invading microorganism. Proteolytic cascades triggered by microbial determinants lead to the formation of melanotic clots at the site of infection (22). The toxic melanin along with encapsulating lamellocytes that circulate in the Drosophila hemolymph can neutralize many foreign microorganisms (23, 24). Other hemocytes actively phagocyte invading pathogens (25, 26). In addition, pathogenic determinants acti-
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Variate Drosophila pattern recognition receptors, which initiate signal transduction cascades that trigger a humoral immune response. This response is marked by the production of antimicrobial peptides (AMPs) that have activity against the invading pathogen. Two key pathways, named Toll and IMD, have been identified that mediate AMP expression (27–29). The former recognizes primarily fungal or Gram-positive bacterial determinants by upstream pattern recognition receptors (30–32). The activation of the Toll receptor due to detection of primally fungal or Gram-positive bacterial determinants by upstream pattern recognition receptors (30–32). The activation of the Toll pathway leads to the phosphorylation of several adaptor proteins that culminate in the phosphorylation of Cactus, an IkB homologue in Drosophila (33–35). This leads to degradation of Cactus, freeing the NF-κB proteins Dorsal and Dif to translocate to the nucleus. There they activate transcription of a variety of genes important for the immune response, including the antimicrobial peptide Drosomycin (36, 37). In the IMD pathway, activation of the receptor peptidoglycan recognition protein LC (PGRP-LC) by Gram-negative bacterial peptidoglycan (38, 39) leads to the phosphorylation of the adaptor Imd (40) and subunits of Drosophila IKK (DmIKKβ and Kenny/IKKy) (41, 42) and finally the cleavage of the NF-κB-like protein Relish. Relish is responsible for the transcription of many proteins important for the immune response, including the antimicrobial peptide Diptericin (43, 44). Drosomycin and Diptericin are often used as target genes to assay for the activation of the Toll and IMD pathways, respectively.

Here we report evidence that Destruxin A suppresses the Drosophila humoral immune response. We used cDNA microarrays and quantitative PCR to examine the effect of Destruxin A on adult Drosophila gene expression. The data revealed a significant proportion of AMP genes were down-regulated, suggesting that Destruxin may be suppressing components of the Drosophila immune system. The data further showed Destruxin had the ability to lower the expression of AMPs even when an immune response had been activated by Gram-negative bacterial infection. Destruxin also increased susceptibility of the fly to bacterial infection. The susceptibility could be rescued by ectopic expression of components of the IMD pathway. This result suggests that Destruxin mediates the specific down-regulation of AMPs through targeting a Drosophila innate immune signaling pathway and is the first evidence of such a phenomenon in vivo. In the evolutionary arms race between insect and fungus, Destruxins may thus be playing a novel role in facilitating fungal survival through specific suppression of host immune response components.

EXPERIMENTAL PROCEDURES

Spotted Microarray Construction and Analysis—From previous Affymetrix chip-based microarray experiments and a survey of the literature, we selected 464 genes important for Drosophila immune responses. We used Primer3 software to design primers to amplify unique regions of the selected genes, generating fragments between 200–600 bp in length (specific primer sequences can be obtained upon request). Fragments were amplified from whole genomic DNA of wild-type, Oregon R strains of D. melanogaster in a 96-well format. The reaction mixture to produce each amplicon contained 50 ng of Drosophila genomic DNA, 1 μM forward primer, 1 μM reverse primer, 1× Titanium Taq (Invitrogen), and 0.5 mM dNTP. The following PCR protocol was used. An initial 95 °C denaturation step for 5 min followed by 20 cycles of 30 s of denaturation at 95 °C, 30 s of annealing at 60 °C, and 45 s of extension at 75 °C. PCR products were run on agarose gels to confirm amplification success and specificity.

Printing, hybridization, and scanning of slides were performed with an Affymetrix 417 Arrayer and 418 Scanner at the University of Maryland Biotechnology Institute Microarray Core facility located at the Center for Biosystems Research. PCR products were spotted in triplicate on poly-L-lysine-coated glass slides with a mean spot diameter of 100 μm and a spot spacing of 375 μm. Following printing and cross-linking, slides were washed with 1% SDS to remove background, treated with blocking solution (0.2 m succinic anhydride, 0.05 m sodium borate, prepared in 1-methyl-2-pyrrolidinone), and washed with 95 °C water and 95% ethanol. After drying, slides were kept in the dark at room temperature.

For microarray experiments, RNA was extracted from a pooled sample of 20 flies with STAT-60 buffer, according to the manufacturer’s protocols (Isotex Diagnostics). They were further purified using the Qiagen RNeasy purification kit and directly labeled using the Cyscribe first strand labeling kit (Amersham Biosciences), according to the manufacturer’s protocols. The raw scanned image files were analyzed using Spotfinder (TIGR), and data normalization, quality assurance and control, filtering, and clustering were performed using MIDAS (TIGR) and MS-Excel (45). Standard deviation normalization and Lowess transformation was performed on the data using MIDAS software. Experiments were done in triplicate, and genes that had at least two readable spots were selected. A criterion of one standard deviation above or below the mean induction of all genes was used to select for up- or down-regulated genes. On normalized data, this represents the top (and bottom) 16% of all genes on the array. The genes were then classified according to available gene ontology classifications, and the major groups are presented in Fig. 1.

Fly Stocks—OregonR flies were used as wild type. For ectopic expression of Toll and IMD pathway components, we used the transgenic c564-Gal4 line of flies that express Gal4 in various tissues throughout the fly, particularly in the lymph gland, fat body, salivary glands, imaginal discs, gut, and brain (46). The c564-Gal4 flies were crossed into transgenic flies expressing upstream activating sequence (UAS)-DmIKKβ (provided by K. V. Anderson); UAS-PGRP-SA (provided by J. Royet); UAS-Imd, UAS-Diptericin, and UAS-Drosomycin (provided by B. Lemaître) to ectopically express these components in the fly.

Bacterial Infection, Survival, and Proliferation Assay—Escherichia coli DH5α strains were grown up in LB medium overnight, resuspended in an equal volume of filter-sterilized phos-
TABLE 1

Genes affected upon Destruxin A injection

The genes have been classified according to available gene ontology (GO) classification and have been separated into up-regulated and down-regulated categories. The total number of genes in each category is given next to the gene ontology number in the dark boxes, and the relative percentage of genes up- or down-regulated in each category is also provided.

| BIOLOGICAL PROCESSES | Name | Fold Change |
|----------------------|------|-------------|
| Antibacterial        | GO:0006961 | 17 Total |
| Humoral Response     |      |             |
| **Down Regulated**   |      | 52%         |
| CG1373               | Cecropin C | 0.5756      |
| CG1878               | Cecropin B | 0.5926      |
| CG1367               | Cecropin A2 | 0.5961     |
| CG4740               | Atacin    | 0.6056      |
| CG1365               | Cecropin A1 | 0.6453  |
| CG8175               | Metchnikowin | 0.6705  |
| CG12763              | Dipterinc | 0.699      |
| CG8846               | Thor      | 0.78        |
| CG10363              | TEP IV    | 0.8131      |

**Proteolysis and Peptidolysis**

| **Down Regulated** | GO:0006508 | 42 total |
| CG4096              | 0.699477   |
| CG9733              | 0.790125   |
| CG9455              | 0.793      |
| CG6361              | 0.8045     |
| CG16705             | 0.8065     |
| **Up Regulated**    | 10%        |
| CG5909              | 1.284      |
| CG1857              | 1.444      |
| CG4804              | 1.45       |
| CG7788              | 2.34       |

**Heatshock**

| **Down Regulated** | GO:0009408 | 5 total |
| CG4406              | Hsp27      | 0.798   |
| **Up Regulated**    | 40%        |
| CG6489              | Hsp70-BC   | 1.29    |
| CG4462              | Hsp23      | 1.64    |

**Toll Pathway**

| **Down Regulated** | GO:0008063 | 17 Total |
| CG5974              | Pelle      | 0.79    |
| **Up Regulated**    | 17%        |
| CG1857              | Nec        | 1.44    |
| CG16844             | IM3        | 1.46    |
| CG5490              | Toll       | 1.51    |

**Jak Stat Signaling**

| **Down Regulated** | GO:0007259 | 3 Total |
| CG15154             | Socs36E    | 0.758526|

**Apoptosis**

| **Down Regulated** | GO:006915 | 25 total |
| CG4319              | 0.78      |
| **Up Regulated**    | 12%       |
| CG4280              | Cq        | 1.24    |
| CG4345              | Grim      | 1.26    |
| CG7788              | Ice       | 2.34    |

**Polysaccharide Metabolism**

| **Down Regulated** | GO:0005976 | 11 Total |
| CG13422             | GNPB2     | 0.756    |
| **Up Regulated**    | 18%       |
| CG5008              | GNPB3     | 1.25    |
| CG12780             | 1.4370    |

**Lipid Metabolism**

| **Down Regulated** | GO:0006629 | 7 Total |
| CG6675              | 0.7045    |

CG6271 | 0.76

Spermatogenesis GO:0007283 | 12 Total
Down Regulated 8%
CG15378 Lectin 22C 0.806

**MOLECULAR FUNCTION**

**Peptidoglycan Receptor Activity**

| **Down Regulated** | GO:0016019 | 18 Total |
| CG9681              | 0.62       |
| CG14745             | 0.64       |
| CG4437              | PGRP-LF    | 0.66     |
| CG9681              | PGRP-SB1   | 0.74     |
| CG7496              | PGRP-SD    | 0.75     |
| CG14704             | PGRP-LB    | 0.79     |
| **Up Regulated**    | 5%         |
| CG4432              | PGRP-LC    | 1.40     |

**Serine Protease**

| **Down Regulated** | GO:0004867 | 25 Total |
| CG3604              | 0.75       |
| CG9455              | 0.79       |
| CG5794              | Pelle      | 0.79     |
| **Up Regulated**    | 20%        |
| CG16713             | 1.32       |
| CG12172             | Spn43Ac    | 1.36     |
| CG1859              | 1.37       |
| CG1857              | Nec        | 1.44     |
| CG4804              | 1.45       |

**Serine endopeptidase activity**

| **Down Regulated** | GO:0004252 | 20 Total |
| CG9723              | 0.79       |
| CG6361              | 0.804      |
| **Up Regulated**    | 5%         |
| CG5909              | 1.28       |

**Scavenger Receptor**

| **Up Regulated** | GO:0005044 | 11 Total |
| CG4280              | Cq          | 1.24     |
| CG7244              | 1.43       |

**Others**

| **Down Regulated** | 3%         |
| CG13323             | 0.6627     |
| CG13324             | 0.694      |
| CG9616              | 0.725      |
| CG12821             | 0.74       |
| CG17738             | 0.749      |
| CG7267              | 0.788      |
| CG15126             | 0.799      |
| **Up Regulated**    | 6%         |
| CG15068             | 1.23       |
| CG14073             | 1.24       |
| CG3838              | 1.318      |
| CG1600              | 1.32       |
| CG16794             | 1.394      |
| CG14419             | 1.465      |
| CG13905             | 1.48       |
| CG16836             | 1.49       |
| CG9568              | 1.62       |
| CG14481             | 1.667      |
| CG7629              | 2.409      |
phosphate-buffered saline (PBS). Approximately 0.5 μl of the bacteria was then injected into the abdomens of female adult flies using a pneumatic picopump PV820 (World Precision Instruments) apparatus. A solution of 86 μM Destruxin A (Sigma-Aldrich) in 1× PBS was used for Destruxin injection. For gene expression studies, RNA was extracted 4 h after injection. The experiment was repeated a minimum of three times.

Each survival experiment was performed with at least 20 flies and repeated three times. The total number of flies in each treatment was assessed for survival periodically over a five-day period. The Kaplan-Meier statistical model was used to compare fly survival, and \( p \) values \(<0.05\) were deemed significant.

For the bacterial proliferation assay, ampicillin-resistant \( E.\ coli \) were used. Twenty-four hours post-injection, the flies were anesthetized, surface sterilized by dipping in 95% ethanol, and homogenized in 1 ml of LB medium containing 1% Triton X-100 and 100 μg/ml ampicillin. The homogenized medium was incubated at 37 °C for 1 h, and 50 μl was plated on LB-Amp plates representing colony-forming units in 1/20 of a fly. Colonies were counted following an overnight incubation of the LB-Amp plates at 37 °C. The experiment was independently repeated 10 times for each treatment, and the error bars show standard deviation.

**Phagocytosis Assay**—The assay was performed as described previously (26). Adult flies are injected in the abdomen with fluorescein-labeled \( E.\ coli \) particles (Molecular Probes). After 30 min, trypan blue is injected to quench extracellular fluorescence. If the hemocytes are able to take up the fluorescent particles, the fluorescence can be visualized through the cuticle on the dorsal side of the abdomen. In cells deficient for phagocytosis, the fluorescence of the particles remains outside the phagocyte and is quenched by the trypan dye. This results in reduced visualization of particles inside flies deficient in phagocytosis.

**Quantitative PCR**—RNA was isolated using STAT-60 buffer according to the manufacturer’s protocol (Isotex Diagnostics). The RNA was digested with RNase-free DNase and subjected to reverse transcription using Superscript II (Invitrogen). The resulting cDNA was quantified using real time-PCR using LUX probes (Invitrogen) on an ABI 5700 real time-PCR system following the manufacturer’s protocols. Gene expression was normalized using RP49 as an endogenous control. The specific primers used can be obtained upon request. The experiments were repeated a minimum of three times and in some cases over five times.

**RESULTS**

**Destruxin A Suppresses Drosophila AMP Immune Response**—To test the effect of non-lethal doses of Destruxin on \( Drosophila \) gene expression, we compared wild-type flies injected with 86 μM Destruxin A to flies injected with PBS using cDNA microarrays. The dose was determined experimentally as the highest dose that could be injected into the fly without causing significant difference in mortality compared with PBS-injected flies within five days (data not shown). The custom-made microarrays enabled the study of 464 \( Drosophila \) genes selected from an extensive literature survey of data collected by other groups through microarray experiments on genes predicted to be important for the immune response (28, 47). Genes that were significantly up- or down-regulated upon Destruxin injection were classified based on known gene ontology (GO) information (Table 1).
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FIGURE 2. Quantitative real time-PCR looking at the effect of Destruxin on specific antimicrobial peptides over time. Co-injecting Destruxin with E. coli causes a lowering of Drosomycin, Diptericin, and Attacin gene expression compared with E. coli-injected flies within 4 h of injection. Adult flies more than five days old were injected with PBS (solid gray), E. coli (solid black), 86 μM Destruxin (dashed gray), or E. coli + Destruxin (dashed black). RNA was isolated from pooled samples of 20 flies 4, 8, and 24 h after injection, and quantitative real-time-PCR was done to examine gene expression. The data were normalized using Drosophila RP49 as an endogenous control, and the y-axis represents relative expression compared with un.injected transcript levels set as 1. The experiment was repeated at least three times and in some cases over five times. The error bars represent S.D. The error bars are only shown for the E. coli- and E. coli + Destruxin-injected flies. Statistically significant differences were assessed at all time points using an unpaired one-tailed Student’s t test. Asterisks are used to denote significant differences (p < 0.05) between PBS and E. coli (gray) and between E. coli and E. coli + Destruxin injected flies (black).

(minimal information about a microarray experiment)-compliant raw data can be accessed from the NCBI GEO website (accession number GSE5767). Categories of genes for general metabolism were not affected by Destruxin. By contrast, 52% of all antibacterial humoral response genes (GO:0006961) on the array were down-regulated in Destruxin-injected flies (Table 1). This represents nearly 23% of all down-regulated genes on the microarray (Fig. 1B) and represents the largest category in the chart. In these microarray experiments, any observed change in gene expression is presumably because of the effect of Destruxin (signal) or noise in the microarray system compared with gene expression in the control. If it is all noise, using a one-standard-deviation criterion would result in ~15% of genes down-regulated in any given gene ontology category on normalized microarray data assumed to follow a normal distribution of gene expression. This means that, of the 17 antibacterial humoral response genes represented on the microarray, we would expect to see 2.5 genes down-regulated by chance alone. However, we observed nine. A y-square analysis with one degree of freedom shows that this observed value is significantly different from the expected 2.5, with a p value of 0.0343. In addition, none of the genes in this category were found to be induced upon Destruxin injection (Fig. 1A and Table 1). Thus, there is a statistically significant down-regulation of antibacterial humoral response genes.

Similarly, 34% of genes having peptidoglycan receptor activity (GO:0004867) were down-regulated, representing 15% of all down-regulated genes (Fig. 1B). Only 5% of genes in this category were induced (Table 1). Proteolysis and peptidolysis genes (GO:0006508) represented the largest percentage of all up-regulated genes (Fig. 1A), but only 10% of all genes in this category were up-regulated (Table 1). Because 11% of these genes were down-regulated, there was no significant shift in either direction for this category (Table 1). Of note, most genes (nearly 85% of the genes on the array) were not affected by Destruxin injection, suggesting that the down-regulated antibacterial humoral genes represent a specific phenomenon and are not the result of general ill health brought about by Destruxin injection. Thus, compared with other categories, we observed the most significant and specific down-regulation of the antibacterial peptide response.

To confirm that Destruxin caused down-regulation of antimicrobial peptide genes, quantitative real-time-PCR was used to examine Diptericin, Cecropin, Attacin, and Metchnikowin expression (Fig. 2). In all cases, the injected flies had a significantly lower AMP production than PBS-injected flies within 4 h of injection (using a one-tailed Student’s t test cutoff of p < 0.05), as predicted by the microarrays. Quantitative real-time-PCR confirmed the suppressive effects of Destruxin on these AMPS in the absence of infection. We used the Gram-negative bacteria E. coli to determine whether Destruxins can also reduce AMP expression when the immune response has been activated. Co-injection of Destruxin A with the bacteria
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FIGURE 3. Co-injection of Destruxin with E. coli causes decreased Drosophila survival. A, thirty flies or more were injected with PBS, E. coli (EC), Destruxin (Drx), or E. coli + Destruxin (EC + Drx), and the number of flies alive in the vial was counted over the course of the next five days. The Kaplan-Meier curve shows significant fly mortality in E. coli-Drx-injected flies but not control animals (p < 0.05 using Mantel-Cox log-rank statistics). B, Destruxin-injected and control flies were assayed for proliferation of live bacteria. Bacterial colony counts show lower bacterial titers upon E. coli injection compared with Destruxin + E. coli-injected flies (p < 0.05 using a two-tailed Student’s t test). Error bars represent S.D. of more than 10 independent experiments.

For 24 h. They were then homogenized in LB medium and spread on LB-ampicillin plates. A count of the resulting colonies revealed a 2–4-fold higher titer of live colonies inside the Destruxin- and E. coli-injected flies compared with flies injected with E. coli alone (Fig. 3B). This observed correlation between bacterial proliferation and fly mortality is consistent with the hypothesis that a lowering in AMP production brought about by Destruxin allows E. coli to proliferate and colonize the fly to accelerate its demise.

Interestingly, Destruxin does not appear to affect Drosophila cellular immune responses in vivo. Destruxin-injected flies continued to demonstrate melanization spots at the site of injection, suggesting that components of the prophenoloxidase cascade remain unaffected (data not shown). Although Destruxins have been shown to affect the ability of hemocytes to phagocytose in cell culture (13, 14), we saw no such effect in vivo in Drosophila. Phagocytosis of E. coli bacterial particles in Destruxin-injected flies appeared to be the same as that seen in PBS-injected flies (Fig. 4). Thus, proliferation of bacteria in the Destruxin-injected flies appears to be a consequence of the lower expression of antimicrobial peptide genes.

Ectopic Expression of Components of the IMD Pathway Rescues Bacterial Susceptibility in Destruxin-injected Drosophila—Antimicrobial peptides are induced through the activation of the Toll and IMD signaling pathways. Thus, we hypothesized that Destruxin might be affecting these signaling pathways to mediate a specific down-regulation of antimicrobial peptide genes. To test this hypothesis, we ectopically expressed various components of the Toll or IMD pathway using a UAS-Gal4 promoter system (48). In this system, we used the Drosophila c564 line to drive expression of a yeast transcription factor Gal4, in various Drosophila tissues, including those that normally express AMP genes, the fat body, and the lymph glands (46). Gal4 protein binds UAS to transcribe genes that have been placed downstream of the UAS regulatory element. In this experiment, components of the Toll and IMD pathway are expressed through

significantly reduced expression of Diptericin, Attacin, and Drosomycin in these immune-stimulated flies at 4 h as compared with injection with E. coli (Fig. 2). To address the question of whether Destruxin was inhibiting or merely delaying the expression of these AMPs, we also examined their expression at 8 and 24 h. For Drosomycin, Destruxin appears to suppress expression at the earlier time point but not at the later time points. Drosomycin expression typically peaks at 24 h after infection, and it is possible that the injected Destruxin is no longer effective at this later time point. Inhibition of Diptericin and Attacin expression by Destruxin was easier to interpret because of the relative transience in their expression. Inhibition by Destruxin also appeared to be specific to these three AMPs, as levels of some others, such as Cecropin, Drosocin, and Metchnikowin, induced by bacterial injection were not significantly affected by Destruxin.

Destruxin-injected Flies Are More Susceptible to Bacterial Infection—We performed survival assays to determine whether the decrease in antimicrobial peptide expression produced by Destruxins leaves flies more vulnerable to microbial infection. Wild-type flies injected with E. coli, Destruxin A, or PBS alone show very low mortality rates. However, co-injection of Destruxin A with E. coli causes a significant decrease in survival compared with control flies, with over 70% dead within five days (Fig. 3A). E. coli is not naturally lethal to the fly, and flies injected with the bacteria alone do not demonstrate significant mortality. To test whether the lower expression of AMP genes also resulted in greater bacterial proliferation within the fly, we performed bacterial survival counts from infected flies. Drosophila were injected with ampicillin-resistant E. coli with or without Destruxin and incubated at 29 °C

FIGURE 4. Phagocytosis assay. Flies were injected with fluorescein E. coli particles, and the signal was quenched 30 min later with trypan blue dye. Any visible fluorescence is a result of phagocytosed bacterial particles. Flies that were injected with control PBS (A) and flies that were injected with Destruxin A (B) show no observable difference in degree of phagocytosis. This suggests that Destruxin injection does not cause a defect in Drosophila phagocytosis responses.
this system independent of their activation by injection. We tested whether the system was working by looking at the expression levels of the target genes Diptericin and Drosomycin (Fig. 5). DmIKKβ and imd expression led to significant levels of Diptericin expression in the absence of infection, and these expression levels continued to remain higher than wild type even upon Destruxin injection. This confirms the activation of the IMD pathway through independent expression of these components (Fig. 5). Diptericin expression in UAS-Imd is inhibited by Destruxin injection, whereas in UAS-DmIKK it is not. This epistatic observation suggests that Destruxin may be acting on a target downstream of Imd but upstream of DmIKK (Fig. 5). In some cases, however, such interpretation was not as straightforward. imd expression, for example, also managed to induce relatively high levels of Drosomycin, a target of the Toll pathway. Similarly, ectopic expression of PGRP-SA, which is known to be upstream of Toll (49) was better at inducing Diptericin than Drosomycin. Thus, there may be cross-talk between the Toll and IMD pathways.

When flies ectopically expressing components of the IMD pathway were co-injected with E. coli and Destruxin, they survived significantly better than wild-type flies given the same treatment (Fig. 6). However, because PGRP-SA weakly activated targets of the IMD pathway (Fig. 5), it was unclear which pathway was responsible for mediating the observed rescue. Expression of PGRP-SA may induce other AMPs and effector genes, and this may also contribute to the observed protection.

To resolve this issue, we ectopically expressed the target anti-microbial peptides Drosomycin and Diptericin. Ectopic Drosomycin expression alone failed to rescue the survival of E. coli and Destruxin co-injected flies (Fig. 6). Drosomycin, an important target of the Toll pathway, has lower expression in Destruxin-injected flies (Fig. 2). However, in the absence of rescue, it remains unclear whether this lowering is mediated through the Toll pathway. On the other hand, the expression of the IMD pathway effector Diptericin was sufficient to significantly rescue survival of flies, although to a lower level compared with DmIKKβ. These results further validate our model that Destruxin-induced Drosophila mortality is because of bacterial proliferation resulting from immune suppression, because the expression of an antibacterial peptide makes them significantly less vulnerable to infection. This also indicates that Destruxin acts upstream of the IMD pathway to mediate a lowering of Diptericin expression.

**DISCUSSION**

Destruxins are secreted by a variety of fungi and are best known for their insecticidal and phytotoxic activity during the establishment of fungal infection (5, 6). They are also gaining utility as lead compounds for the generation of anti-viral and anti-cancer agents for human therapies (16, 20). There is evidence suggesting that their biological role may involve manipulating host signaling (15). We used cDNA microarrays to test the effect of injecting Destruxin A into adult D. melanogaster. Injection of Destruxin may be representative of the physiological scenario, as fungal hyphae secrete it after penetrating the cuticle. Injection also allowed us to isolate the effect of Destruxin on Drosomycin gene expression and survival in a controlled way, independent of other components of a natural fungal infection. Microarray results showed the antibacterial humoral response genes are disproportionately down-regulated compared with other categories of genes, and this was the most significant difference in gene expression patterns. Although the array is enriched for immunity genes, this finding still represents a specific shift in expression of genes compared with PBS-injected controls.

We did not observe any failure in the phagocytosis or melanization machinery in vivo. Thus, Destruxin appears to be specifically affecting components of the humoral immune response. Furthermore, this effect is sufficient to cause greater susceptibility to bacterial infections. This mortality is accompanied by a greater proliferation of E. coli in Destruxin-injected flies, suggesting that the suppression by Destruxin of the immune response genes makes the fly unable to clear a bacterial infection. To explore the mechanism by which Destruxin mediates suppression of AMPs, we ectopically expressed components of the Toll and IMD path-

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**FIGURE 5. Quantitative PCR showing expression of Drosomycin (top) and Diptericin (bottom).** Relative expression of target genes Drosomycin and Diptericin are given, using RP49 as an endogenous control and using uninjected wild-type transcript levels as a calibrator, set to 1. Wild-type-unjected, E. coli-, Destruxin-, and E. coli + Destruxin-injected transcript levels are provided as a reference. Transgenic *Drosophila* ectopically expressing components of the Toll pathway (PGRP-SA) or IMD pathway (imd and DmIKKβ) are given. Ectopic activation of various IMD pathway components activates the IMD target Diptericin compared with Oregon R-uninjected controls in a statistically significant manner (p \(<\) 0.05 for Diptericin expression for UAS-PGRP-SA, UAS-Imd, and UAS-DmIKK). Drosomycin expression is not significantly induced upon ectopic expression of Toll or IMD pathway components. A Student’s t test comparison between samples representing statistically significant changes are shown using a bar (* represents p < 0.05 with a two-tailed Student’s t test). Experiments were repeated at least three times. Error bars represent S.D.
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FIGURE 6. Graphical analysis of survival assay using ectopic expression of Toll and IMD components. The UAS-Gal4 system is used to drive the expression of components of the Toll and IMD pathway. The Drosophila c564 line is used to drive expression of IMD pathway genes UAS-Imd (A), UAS-DmIKKβ (B), Toll pathway genes UAS-PGRP-SA (C), and antimicrobial effectors UAS-Drosomycin (D) and UAS-Diptericin (E). Survival of IMD-, DmIKKβ-, PGRP-SA-, and Diptericin-expressing lines produced a statistically significant rescue compared with wild-type E. coli + Destruxin co-injected flies (p < 0.05). The survival rates of wild-type flies injected with E. coli (solid gray line with •) or with E. coli and Destruxin (dotted gray line with □) transgenic Gal4-UAS flies expressing components of the Toll or IMD pathway injected with E. coli (solid black line with ▲) or with E. coli + Destruxin (dotted black line with ×).

ways. Imd-, DmIKKβ-, and Diptericin-producing flies were significantly less susceptible to mortality from co-injection of E. coli and Destruxin. Because expressing components of the IMD pathway alone facilitates fly survival, Destruxin may suppress the IMD pathway. Because Destruxin fails to lower expression of Diptericin in DmIKK but not Imd-expressing flies (Fig. 5), it may be acting on a target upstream of DmIKK but downstream of Imd. This suppression may be specific to the IMD pathway as Destruxin fails to inhibit metabolic housekeeping genes and other aspects of the immune response. It is not known whether this immune pathway suppression is related to the ability of Destruxin to affect calcium channels and induce paralysis in other insects (11).

Suppression of a host immune response would have obvious benefits for a pathogenic fungus such as M. anisopliae. Flies lacking the ability to produce AMPs due to mutations in both the Toll and IMD pathways are extremely vulnerable to fungal challenge (29, 50), and these innate immune pathways are conserved between insects. Thus, the ability to reduce AMP production is likely to aid fungal survival in a variety of insect hosts.

It is noteworthy that Drosomycin, the only AMP with known inhibitory activity to M. anisopliae, was one of the AMPs suppressed by Destruxin. Insects also seem to have pathways responsible for Destruxin biotransformation (51). Studies with Destruxin E in locusts suggest the existence of pathways that metabolize the toxin and produce a population of secondary compounds (52, 53). This may be indicative of a co-evolutionary history between fungi producing these toxins and insects evolving biochemical means to detoxify them. Recently, Varroa mites were shown to suppress humoral AMP expression in honey bees (54). Similar suppression by fungi-expressing Destruxins adds a new dimension to the dynamics of host-pathogen interactions.

Insect pathogenic fungi have been under-exploited as a resource of medically active compounds, but this is changing with the realization that they are exceptionally rich sources of novel biologically active substances (55). To date, the only commercialized example is cyclosporine, a undecapeptide from the mosquito pathogenic fungi Tolypocladium inflatum that prevents IL-2 expression in mammalian cells by inhibiting calcineurin (56). Cyclosporin A is therefore used extensively in current human therapies such as organ transplantation, which require suppression of the host immune response (57).

Destruxins are also candidates for medical use particularly as therapeutic agents for viral diseases and cancer. But as this study shows, Destruxins have complex effects on cells, and more research is required to predict and improve cellular responses to them.

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