



EXPRESSION OF ANTIMICROBIAL PEPTIDE GENES AFTER INFECTION BY PARASITOID WASPS IN *Drosophila*

Emmanuelle Nicolas,* Anthony J. Nappi† and Bruno Lemaitre*

*Institut de Biologie Moléculaire et Cellulaire, UPR 9022 du CNRS, 15 rue René Descartes, 67084 Strasbourg, Cedex, France

†Department of Biology, Loyola University of Chicago, 6525 North Sheridan Road, IL 60626, Chicago, U.S.A.

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Abstract—We report here the use of a specific β -galactosidase staining assay and Northern blotting technique to examine the expression of three genes encoding either antibacterial peptides (diptericin, cecropin A) or an antifungal peptide (drosomycin) in *Drosophila* following infection by larval and pupal parasitoids. The results show that the genes encoding these peptides are either not induced or minimally induced in wasp-infected hosts, but remain responsive and are induced upon microbial challenge. As the parasitoids elicit a cellular response, our data suggest that the antimicrobial responses are activated and/or regulated by mechanisms that are independent of those mediating cellular encapsulation. Copyright © 1996 Elsevier Science Ltd.

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Introduction

The humoral immune molecules in insects include antibacterial and antifungal peptides, proteases, prophenoloxidase/pheno-

loxidase, and lectins (1–3). The cellular immune reactions consist essentially of phagocytosis and encapsulation by circulating blood cells which are frequently mobilized to form melanotic capsules around foreign entities that are too large to be phagocytosed (4–6). The mechanism leading to the cellular encapsulation of parasites has been well documented at the morphological level (6) but is still poorly understood at the molecular level (7). The inducible antimicrobial peptides that appear in the hemolymph of insects in response to bacterial or fungal challenge are synthesized primarily by the fat body and hemocytes. In *Drosophila*, the genes or cDNAs encoding some of these inducible molecules have been cloned (i.e. cecropins (8,9); diptericin (10); defensin (11); drosocin (12); attacin (13); drosomycin (14)). The mechanism(s) controlling the expression of these antimicrobial peptides after immune challenge is an important question in the field of insect immunology.

The objective of this investigation was to examine the extent to which mechanisms regulating the production of antibacterial peptides and cellular immune components in *Drosophila melanogaster* are co-regulated. To study the possible involvement of antibacterial genes in cellular encapsulation we monitored the

Address correspondence to Dr Bruno Lemaitre, UPR 9022, Institut de Biologie Moléculaire et Cellulaire, 15 rue René Descartes, 67084 Strasbourg Cedex, France.

inducible expression of two antibacterial peptides and one antifungal peptide in *Drosophila melanogaster* infected by the wasp parasitoids *Leptopilina bouleari*, *Leptopilina heterotoma*, or *Trichopria sp.*, which oviposit a single egg into the body cavity of *Drosophila* larvae or pupae, respectively.

Materials and Methods

Drosophila Stocks

Oregon R flies were used as a standard wild-type strain. The transgenic strain, Dipt2.2-*lacZ*:1 is a ry⁵⁰⁶ C.S. line carrying a dipterocin reporter gene on the X chromosome (10). The fusion gene containing 2.2 kb of dipterocin upstream sequences fused to the bacterial *lacZ* coding region was inserted into the Carnegie 20 vector (15). The developmental and inducible expression of the Dipt2.2-*lacZ* transgene has been previously described (10). The inducible expression of the Dipt2.2-*lacZ* is superimposable to that of the resident dipterocin gene at the end of the third larval stage.

All experiments were performed at 25°C unless otherwise stated.

Origin of Wasp Strains and Infection Procedures

The parasitic wasps *L. bouleari*, *L. heterotoma*, and *Trichopria sp.* used in this study were raised at 25°C on a wild-type strain of *D. melanogaster* (Oregon R). *Leptopilina* species lay their eggs inside *Drosophila* larvae. The egg hatches after approximately 48 h and the young wasp larva develops to the adult stage and emerges as an adult from the host puparium after 18–20 days. *Trichopria* lay eggs inside 2-day-old pupae and emerge as adults after 18 days.

Drosophila females were provided a 6-h

oviposition period on standard medium. Second stage *Drosophila* larvae (approximately 50 h) were exposed for 8 h to *Leptopilina* females that had not previously oviposited (4). To ensure adequate numbers of parasitized larvae and to minimize multiparasitism, five female parasitoids were used per 200–300 larvae. Under these conditions, 80% of the larvae were infected. From 48 to 72 h after parasitization, larvae were collected and divided into two batches. The first group was dissected to determine the percentage of parasitization, and the second group was used to evaluate gene expression. A similar procedure was followed when 2–4-day-old pupae were exposed to *Trichopria*.

Injury Experiments

Injury experiments were performed by pricking flies with a needle that had been previously dipped in a concentrated bacterial culture of *Escherichia coli* and *Micrococcus luteus*. The majority of bacterially challenged larvae died during the pupal stage.

Quantitative Measurement of β -Galactosidase Activity

The procedure described by Lemaitre and Coen (16) was applied to homogenates made from groups of five individuals of either third stage larvae or pupae. Results are given in nanomoles of product formed/(min mg⁻¹) protein.

β -Galactosidase Localization

The fat bodies were fixed for 5 min in PBS, pH 7.5, containing 1% glutaraldehyde and 1 mM MgCl₂ (17). Subsequently, tissues were washed in PBS and immersed in 0.2% 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal),

3.5 mM $K_4Fe(CN)_6$, 3.5 mM $K_3Fe(CN)_6$, 1 mM $MgCl_2$, 150 mM NaCl, 10 mM Na_2HPO_4 , 10 mM NaH_2PO_4 and incubated for 4 h at 37°C. The tissues were then mounted in glycerol.

RNA Expression of Genes Encoding Antimicrobial Peptides

Total RNA extraction and Northern blotting experiments were performed with tissues removed from control, bacteria-challenged, and parasitized *Drosophila* using methods described previously (18). The following probes were used to detect RNA expression: dipterecin cDNA (19), drosomycin cDNA (14), rp49 cDNA, (a PCR fragment of approximately 400 bp generated between two oligonucleotides designed after the rp49 coding sequence (20)) and a 21-mer oligonucleotide (5'-GATTCCCAGTCCCTGGATTGT-3') complementary to part of the coding sequence of cecropin A1 which is identical for cecropin A2 (8).

Results

Analysis of the Expression of a Dipterecin-lacZ Reporter Gene in Transgenic Parasitized *Drosophila*

For our experiments we have used the transgenic Dipt2.2-lacZ:1 strain, which has stably integrated a lacZ reporter gene fused to the promoter (2.2 kb) of the gene encoding the antibacterial peptide dipterecin. A previous study has shown that the induction of this reporter by immune challenge parallels that of the resident dipterecin gene (10). In initial experiments the transgenic strain Dipt2.2-lacZ:1 was found to be totally susceptible to the parasitoids, there being no evidence of a successful cellular encapsulation response against either species of *Leptopilina* or *Trichopria* (data not shown).

We have first investigated the expression of the Dipt-lacZ reporter gene in larvae infected by *Leptopilina bouhardi* and *Leptopilina heterotoma*. For this, second stage *Drosophila* larvae carrying the transgene were parasitized and assayed for β -galactosidase activity 96 h post-infection (Table 1). In contrast to non-parasitized bacteria-challenged larvae, no expression of the Dipt2.2-lacZ reporter gene was observed in infected larvae. Thus, neither *L. bouhardi* nor *L. heterotoma* elicited a marked Dipt2.2-lacZ:1 expression.

To exclude the possibility that the absence of expression of the Dipt2.2-lacZ:1 transgene in wasp-infected second instar larvae reflected the low level of inducibility of the reporter gene at this stage of development (10), we have parasitized older, third stage larvae (approximately 96 h) with *L. bouhardi*. At 6-h post-infection, the fat body was removed from these hosts and stained for β -galactosidase activity (X-gal staining). Little or no β -galactosidase activity was observed in parasitized larvae or in non-parasitized control larvae. In contrast, a deep blue coloration was apparent in fat body cells of parasitized larvae that were

Table 1. Dipt-lacZ2.2 Activity in Wasp-infected Larvae

Larvae	Number of measurements	β -galactosidase activity
(-)	7	1.6 \pm 1.4
(+)	8	83.0 \pm 28.5
L.h.(-)	8	1.1 \pm 1.6
L.h.(+)	8	92.2 \pm 41.3
L.b.(-)	8	1.3 \pm 0.8
L.b.(+)	8	107.6 \pm 58.5

Results are the number of measurements, mean and the confidence interval ($p < 5\%$). Activity was measured in a pool of five control or wasp-infected wandering larvae that were either unchallenged (-) or sacrificed 3 h after bacterial challenge (+). The endogenous level of β -galactosidase was obtained by measuring the β -galactosidase activity in ry^{506} sibs of the Dipt2.2-lacZ:1 strains, devoid of Dipt2.2-lacZ:1 insertion. It was 0.9 ± 0.2 ($n=8$). L.h.: *L. heterotoma*; L.b.: *L. bouhardi*.

subsequently challenged with bacteria (data not shown). In separate experiments, quantitative measurements of β -galactosidase activity were made of homogenates of five third instar parasitized and bacteria-challenged larvae. These experiments showed levels of reporter gene expression that were similar to those obtained from non-parasitized, bacteria-challenged larvae (see Table 1). These observations indicate that parasitized larvae are not physiologically compromised and are fully capable of eliciting a wild-type induction of the dipterin reporter gene in response to bacterial challenge.

We next studied the expression of the dipterin reporter gene in *Drosophila* pupae parasitized by *Trichopria*. For these investigations, 2-day-old pupae from the Dpt2.2-*lacZ*:1 stock were used as hosts and exposed to parasitoids for 8 h. A low but significant level of reporter gene expression was observed 24 h after *Trichopria* infection (Table 2). However, the level of Dpt2.2-*lacZ*:1 expression was significantly lower and induction kinetics slower than those observed in non-parasitized, bacteria-challenged pupae. In

addition, following bacterial challenge, the Dpt2.2-*lacZ*:1 reporter gene was found to be fully inducible in pupae infected by *Trichopria* (Table 2).

RNA Expression of Genes Encoding Antimicrobial Peptides in Parasitized *Drosophila*

Northern blot analysis was used to monitor the expression of various genes encoding antimicrobial peptides after infection by wasp parasitoids. RNAs from control, parasitized, and both parasitized and bacteria-challenged larvae or pupae were extracted. The Northern blot filter was successively probed for the dipterin, cecropin A and drosomycin genes. A probe corresponding to the rp49 gene which encodes a ribosomal protein, was also used as a control for the amount of RNA.

The genes encoding the antibacterial peptides dipterin or cecropin A were not expressed in unchallenged larvae and pupae (Fig. 1), but a detectable level of expression was monitored for the antifungal peptide drosomycin, corroborating previous published data in the literature (8,10,14). High levels of expression were observed for all three immune genes in bacteria-challenged insects (Fig. 1). In agreement with the results obtained above with the transgenic strain, there was little or no expression of these immune genes in wasp-infected individuals. In parasite-infected larvae and pupae, there was considerable variation in gene expression. Cecropin A activity was not induced by any parasitoid. The dipterin gene was expressed in individuals parasitized by *L. boulardi* and by *Trichopria*. The antifungal gene encoding drosomycin was induced by *L. heterotoma* and *Trichopria*. However, radioactivity measurements indicated that the level of induction after wasp-infection was always significantly lower than after challenge by bacteria [(Fig. 1), data not shown]. Wild-

Table 2. Dpt2.2-*lacZ*:1 Activity in *Trichopria*-Infected Pupae

Pupae	Number of measurements	β -galactosidase activity
(-)	13	2.6 \pm 2.6
(+)	8	51.5 \pm 18.9
6 h P.I.*(-)	8	15.5 \pm 6.4
14 h P.I.(-)	8	11.5 \pm 5.5
14 h P.I.(+)	8	57.8 \pm 20.0
24 h P.I.(-)	8	19.7 \pm 6.7
48 h P.I.(-)	8	13.6 \pm 6.8

Results are the number of measurements, mean and the confidence interval ($p < 5\%$). Activity was measured in a pool of five control or wasp-infected pupae that were either unchallenged (-) or sacrificed 6 h after bacterial challenge (+). The endogenous level of β -galactosidase was obtained by measuring the β -galactosidase activity in ry^{506} sib pupae of the Dpt2.2-*lacZ*:1 strains, devoid of Dpt/*lacZ* insertion. It was 5.4 \pm 1.3 ($n=4$). P.I.: post-infection.

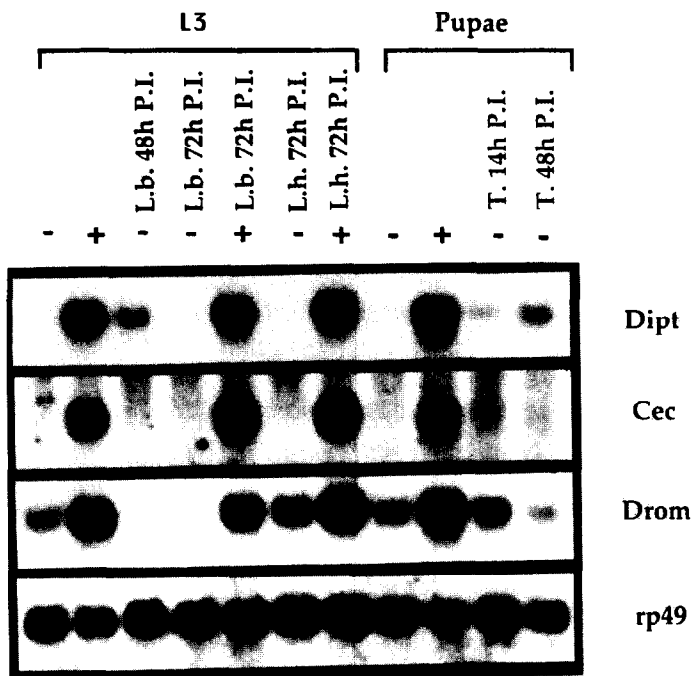


Figure 1. Transcriptional profiles of dipterin, cecropin and drosomycin in wild-type, parasitized and parasitized plus challenged larvae and pupae. Total RNA was extracted at different time intervals (as indicated) after parasitization from wild-type Oregon^R larvae and pupae. Twenty microgram samples were fractionated by denaturing 1% agarose/formaldehyde gel electrophoresis, transferred onto a nylon membrane and successively hybridized with a nick-translated dipterin (Dipt) and drosomycin (Drom) cDNA probe; an end-labeled oligonucleotide probe complementary to cecropins A1 and A2 (CecA) transcripts and a nick-translated rp49 cDNA probe. —: unchallenged animals; +: 6 h challenged animals; L.b.: larvae parasitized by *L. bouleari*; L.h.: larvae parasitized by *L. heterotoma*; T: pupae parasitized by *Trichopria*; L3, wandering third instar larva; P.I.: post-infection.

type levels of induction of all three immune genes were exhibited in wasp-infected larvae after bacterial challenge.

Discussion

Drosophila susceptible hosts were examined for evidence of parasite-mediated induction of three genes encoding antimicrobial immune peptides (dipterin, cecropin A, and drosomycin) following infection by either the larval (*L. bouleari* and *L. heterotoma*) or the pupal stage (*Trichopria*) parasitoids. We found no evidence for strongly induced antimicrobial immune responses in parasitized *Drosophila* using either specific β -galactosidase titration or Northern blotting techniques. These data indicate that eggs

ovoposited in the larvae do not induce a full humoral antimicrobial response. Despite the low level of antimicrobial gene expression in wasp-infected *Drosophila*, the three antimicrobial genes remained fully inducible and were expressed upon subsequent bacterial challenge. These observations strongly suggest that the eggs ovoposited in the larvae are not recognized by the host's humoral mechanism. In contrast, it has been shown that in response to wasp infection, larvae elicit a cellular response (reviewed in Ref. 21). Rizki and Rizki showed that infection by *Leptopilina* wasps induces lamellocyte differentiation in larvae (22). Lamellocyte are large flattened hemocytes derived from plasmatocytes which are involved in the formation of the capsule (21). This last observation indicates that

the presence of the parasitoid is recognized by the immune system of the host. This cellular response is, however, later selectively incapacitated in susceptible larvae (23). Indeed as shown for *L. heterotoma*, the number of lamellocytes from infected larvae decreased, probably due to a destructive factor derived from the accessory gland of the female wasp's reproductive system (24). Taken together, the results of this study indicate that, while altering the cellular response, infection of *Drosophila* by wasp parasitoids does not affect the host's humoral immune mechanism. This last result suggests that the humoral and cellular immune mechanisms may be activated by different mechanisms.

The observations made in this study using two species of *Leptopilina* support in part the recent investigations of Coustau et al. (25) who likewise were unable to detect the induction of antibacterial peptides in immune-reactive (resistant) *Drosophila* infected with *L. boulardi*. This result demonstrates that the presence of antimicrobial peptides is not required for successful encapsulation of the parasitoid (25). However, these authors detected an elevated antibacterial activity in susceptible hosts infected by *L. boulardi*. (25). In contrast, with the three different parasitoid species tested here, only a low level of antimicrobial expression in both parasitized *Drosophila* larvae and pupae was observed. This was attributed to bacterial contamination which occurred during parasitoid oviposition. The combined data suggest that infection of *Drosophila* by wasp parasitoids does not diminish the host's antimicrobial immune response, and that this humoral mechanism is regulated independently of the host's cellular immune system. The

existence of different recognition mechanisms does not preclude that the cellular and humoral responses share common regulatory elements. This was recently suggested by two reports indicating that rel proteins and the Toll receptor may be involved in both the control of antimicrobial genes and lamellocyte differentiation (18,26).

As with other organisms, insects have evolved with different adaptive responses to various types of aggression (i.e. infection by metazoan parasitoids, bacteria, or fungi). The results of this investigation suggest that, in *Drosophila*, the humoral response elicited by microbial agents is regulated by mechanisms that differ from those which control cellular encapsulation. Additional evidence to support this proposal comes from recent studies showing the differential induction of antibacterial and antifungal genes (27). An important goal of current research in the field of insect immunity is to decipher the specific recognition mechanism(s) for pathogens that trigger the immune defense.

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